Neutrophil antimicrobial proteins enhance *Shigella flexneri* adhesion and invasion

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Zusammenfassung

Shigella flexneri verursacht im Verlauf der Infektion eine massive Enzündungsreaktion sowie Schädigung des humanen Darmepithels. Neutrophile sind die ersten Zellen des angeborenen Immunsystems, welche den Infektionsherd infiltrieren. Diese Zellen greifen Mikroorganismen mittels Phagozytose, Neutrophiler extrazellulärer Fallen (Neutrophil Extracellular Traps, NETs) oder Degranulierung an. In dieser Arbeit haben wir untersucht, wie die Degranulierung von Neutrophilen die Virulenz von Shigellen beeinflußt und konnten zeigen, dass die Exposition von Shigellen mit Proteinen aus den Granula von Neutrophilen die Invasion in Epithelzellen stark erhöht. Während dieser Exposition binden kationische Proteine der Granula an die Oberfläche von *Shigella* und bewirken eine verstärkte Adhesion, welche dann schließlich zu “Hyperinvasion” führt. Dieser Effekt wird durch Änderungen der Oberflächenladung bewirkt, da eine Lipopolysaccharid (LPS) Mutante mit negativer Oberflächenladung eine zusätzliche erhöhte Hyperinvasion im Vergleich zu Wildtyp Shigellen zeigt. Zusätzlich zur Hyperinvasion bewirkt die Infektion von Epithelzellen mit Shigellen, die mit Granula Proteinen in Kontakt gekommenen sind, eine Verminderung der IL-8 Sekretion. Dieses Zytokin bewirkt eine starke Rekrutierung von Neutrophilen. Daher stellen wir die Hypothese auf, dass *Shigella* in der Lage ist, antimikrobielle Proteine des Wirtes zur Erhöhung seiner Virulenz durch Hyperinvasion zu verwenden sowie eine weitere Rekrutierung von Neutrophilen durch Inhibition der IL-8 Sekretion zu verhindern. Somit unterwandert *Shigella* das angeborenen Immunsystem und nutzt dessen Angriff zu seinem Vorteil.

Shigella

Neutrophile

Antimikrobielle Proteine

Invasion

Abstract

Shigella flexneri is an enteric pathogen that causes massive inflammation and destruction of the human intestinal epithelium. Neutrophils are the first cells of the innate immune system recruited to the site of infection. These cells can attack microbes by phagocytosis, Neutrophil Extracellular Trap (NET) formation and degranulation. Here, we investigated how neutrophil degranulation affects virulence and show that exposure of *Shigella* to granular proteins enhances infection of epithelial cells. During this process, cationic granular proteins bind to the *Shigella* surface causing increased adhesion which ultimately leads to hyperinvasion. This effect is mediated by changes in the surface charge, since a lipopolysaccharide (LPS) mutant with a negative surface shows enhanced hyperinvasion compared to wild-type *Shigella*. In addition, infection with *Shigella* exposed to granular proteins leads to the inhibition of secretion of the neutrophil attracting cytokine IL-8. We propose that *Shigella* uses host defense molecules to enhance its virulence by increased infection of its host cells and reduced recruitment of neutrophils after hyperinvasion through inhibition of IL-8 secretion. With this *Shigella* subverts the innate immune system and uses its attack for its own benefit.

Shigella

Neutrophils

Antimicrobial proteins

Invasion

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1 Introduction

1.1 *Shigella*

1.1.1 Shigellosis

Shigella spp. is the causative agent of bacillary dysentery. Dysentery is characterized by painful abdominal cramps, nausea, fever, tenesmus and frequent stools containing blood and mucus (Maurelli and Sansonetti, 1988). *Shigella* infections are normally self limiting and cleared in about 5-7 days. However, dehydration and subsequent nutrient loss make Shigellosis a dangerous threat for children and older people, especially in developing countries where *Shigella* is endemic and antibiotic treatment is rarely available. About 100 million infections occur per year worldwide resulting in about 1 million deaths (Kotloff, et al., 1999).

Four *Shigella* strains (S. *flexneri*, *S. boydii, S. sonnei* and *S. dysentery*) with different geographical distribution exist, while each strain comprises several serotypes. S. *flexneri* is the most prevalent strain in developing countries, accompanied by occasional isolations of *S. boydii* and *S. sonnei* from patients. *S. dysentery* is rarely isolated in Europe and the United States, but can cause fatal epidemics (Hale, 1991) (Maurelli and Sansonetti, 1988) (Lindberg and Pál, 1993). *S. sonnei* is more prevalent in these countries and is characterized by a less severe clinical progression. *Shigella* is transmitted via the fecal-oral route and is highly infectious. An inoculum of 10-100 bacteria causes dysentery in fifty percent of human volunteers (DuPont, et al., 1989). The symptoms of Shigellosis result from the invasion of the colonic mucosa by *Shigella*. Histopathologic analysis of the colon of patients with shigellosis reveals destruction of the epithelium, mucosal erosion and infiltration of neutrophils (Anand, et al., 1986), (LaBrec, et al., 1964), (Mathan and Mathan, 1991). *Shigella* induces acute

inflammation during infection, which is responsible for the ensuing symptoms (Lindberg and Pál, 1993; Zychlinsky and Sansonetti, 1997).

1.1.2 Pathogenesis

Shigella infects the mucosa of the large intestine, where it breaches the epithelial barrier by traversing through Microfold cells (M-cells) (Wassef, et al., 1989). These specialized cells are located in the Peyers patches and constantly sample antigens from the lumen of the intestine. Since *Shigella* is incapable of invading enterocytes through their apical membrane (Mounier, et al., 1992), M-cells are their only port of entry across the epithelium (Figure 1). Once reaching the basolateral side of the epithelium, resident macrophages phagocytose *Shigella*. *Shigella*, however, is able to escape from the phagolysosome into the cytoplasm using its virulence factors (Sansonetti, et al., 1986). This leads to induction of cytotoxicity, which was described initially described as apoptosis (Zychlinsky, et al., 1992). The presence of *Shigella* in the cytoplasm causes the activation of caspase-1 (Chen, et al., 1996), a cystein protease which processes the proinflammatory cytokines IL-1β and IL-18 into their active forms (Thornberry, et al., 1992). This processing is required for the secretion of both cytokines. After elimination of the macrophages, *Shigella* invades non-phagocytic epithelial cells from the basolateral side by inducing its phagocytosis (Figure 1). This process causes extensive rearrangement of the cytoskeleton of the host cell (Sansonetti and Egile, 1998). *Shigella* enters the epithelial cell by a so-called triggering mechanism involving cellular extensions protruding around the bacterium, achieved by the recruitment of cellular factors by *Shigella* virulence factors (Nhieu, et al., 2005). Induction of phagocytosis in epithelial cells is a key event during infection: Epithelial cells don't inhibit intracellular growth of the bacterium and provide protection against cells of the immune system. Later during infection, intracellular growth of *Shigella* in epithelial cells leads to cell death although this takes

place after much longer time than in macrophages and is not dependent on caspase-1 (Carneiro, et al., 2009). Death of epithelial cells then leads to destruction of the epithelial structure during Shigellosis.

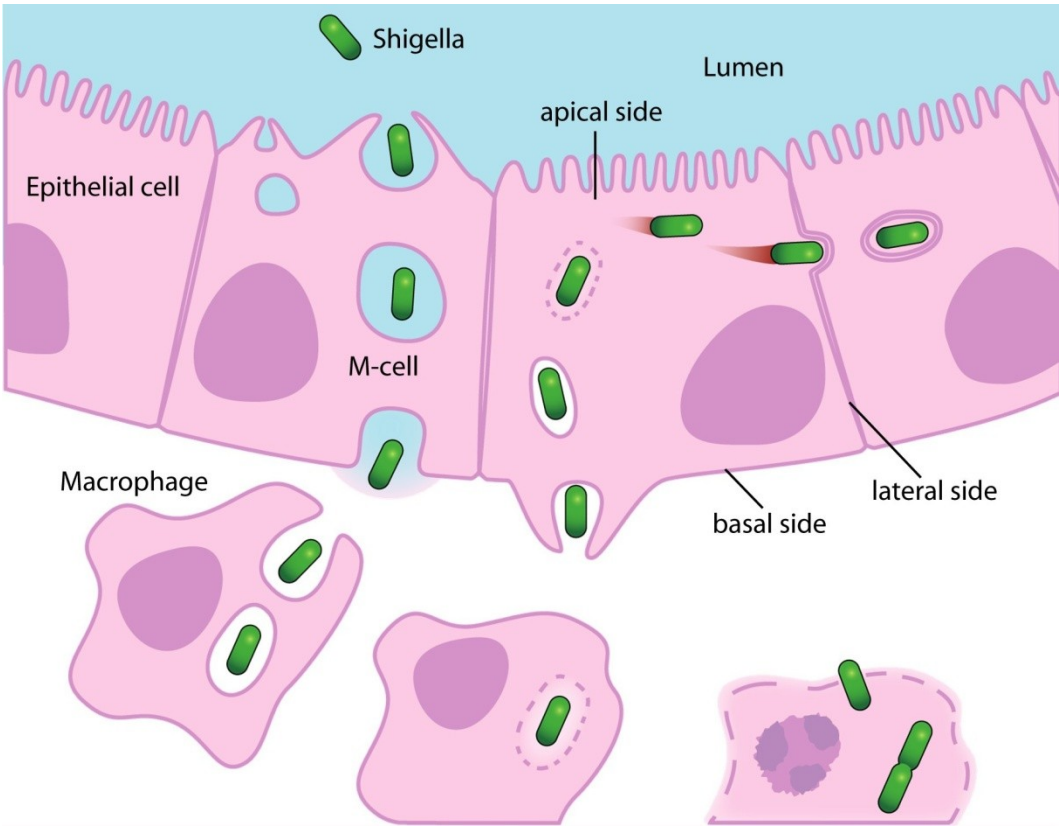

Figure 1: *Shigella* pathogenesis

After bypassing the epithelial barrier through transcytosis of M-cells, *Shigella* is phagcyotosed by resident macrophages. These macrophages are killed by *Shigella* through activation of caspase-1 after *Shigella* escaped from the phagosome. This leads to release of mature IL-1β and IL-18. Then, *Shigella* infects epithelial cells from the basolateral side. It again leaves the phagosome and uses the cellular actin cytoskeleton to propel itself through the cell and into adjacent cells.

1.1.3 Type Three Secretion System

Shigella uses a so called Type Three Secretion System (TTSS) to secrete its virulence factors. The TTSS is a membrane bound secretion system with homology to the flagellar system (Van Gijsegem, et al., 1995). It spans the inner and the outer membrane of certain gram-negative bacteria with its basal body. The basal body is composed of two rings, one of each in one of the membranes. Connected to the basal body is the needle, which is composed of polymerized MxiH monomers. The needle itself has a length of 45 nm (Tamano, et al., 2000) an inner diameter of 2-3 nm (Blocker, et al., 2001) which only allows passage of unfolded proteins. The length of the needle together with the structure of the LPS on the bacterial surface can influence the virulence of *Shigella* (West, et al., 2005). The genes encoding the proteins which form the TTSS are located within two operons and are called Mxi and Spa (for Membrane expression of Ipa and surface presentation of Ipa) (Sansonetti and Egile, 1998).

1.1.4 Virulence factors

In *Shigella*, the genes encoding virulence factors and the TTSS are located in a pathogenicity island (Maurelli, et al., 1985). This island is called entry-region, since it contains all genes required for successful entry of *Shigella* into epithelial cells. Pathogenicity islands - such as the entry region - often encode virulence associated genes and are characterized by a GC content differing from the remaining genome of the bacterium (Hacker and Kaper, 2000). This is believed to be a consequence of horizontal gene transfer between different species.

A first wave of effectors secreted through the TTSS during infection consists of so called Ipa proteins for Invasion Plasmid Antigen (Ipa). These effectors are expressed at 37°C but stay in the bacterial cytosol. Only upon host cell contact or other signals such as serum or the small amphy-

pathic dye congo red they are secreted by the TTSS (Bahrani, et al., 1997; Menard, et al., 1994).

The different Ipa proteins fulfill many functions during pathogenesis: IpaB is required for caspase-1 activation in macrophages as well as for escape from the phagosome in both macrophages and epithelial cells (High, et al., 1992; Zychlinsky, et al., 1994). IpaC and IpaD together with IpaB are also essential for entry of *Shigella* in epithelial cells (Menard, et al., 1993).

Shigella also secretes a second wave of effectors. Expression of these effectors is regulated by IpgC, the cognate chaperone of IpaB and IpaC (Menard, et al., 1994) . Once IpaB and IpaC are secreted, IpgC is released and binds to MxiE (Mavris, et al., 2002). MxiE then acts as a transcriptional regulator, inducing the expression and secretion of IpaH, Osp and other effector proteins. In fact, this regulated expression is needed to organize the secretion into these two waves. Whereas the Ipa proteins are required for entry into epithelial cells and caspase-1 activation in macrophages, the second wave effectors – such as OspF and OspG - are either of unknown function or downregulate the proinflammatory answer of the host following successful infection (Arbibe, et al., 2007; Kim, et al., 2005).

1.2 *Shigella* induced Inflammation

1.2.1 NF-kB activation by *Shigella*

Intracellular *Shigellae* lead to the activation of NF-kB in epithelial cells (Philpott, et al., 2000). This in turn causes expression and secretion of proinflammatory cytokines such as IL-8 (Philpott, et al., 2000). Several intracellular pattern recognition receptors exist in cells to detect the presence of invading microorganisms. One group of these receptors is the Nod proteins (Chen, et al., 2009). These proteins are characterized by a leucine-rich-repeat (LRR), a nucleotide oligomerization domain (NOD) and a caspase activation and recruitment domain (CARD). Nod1 detects-γ-

glutamyl-meso-diaminopimelic acid (iE-DAP) (Chamaillard, et al., 2003; Girardin, et al., 2003), a molecule present in the membrane of gram-negative bacteria. Nod2 recognizes muramyl-dipeptide (MDP) (Girardin, et al., 2003; Inohara, et al., 2003) which is present in gram-positive and gram negative bacteria. iE-DAP and MDP from S. *flexneri* activate both Nod1 and Nod2 and cause their oligomerization. These oligomers recruit and activate the RIP-like interacting CLARP kinase (RICK) - also called receptor-interacting protein2 (Rip2) - by CARD-CARD interactions (Inohara, et al., 2000).

After sensing their respective ligand, both Nod1 and Nod2 activate RICK by inducing its K63-polyubiquitination (Hasegawa, et al., 2008). In the case of Nod2, this ubiquitination is recognized by the transforming growth factor beta-activated kinase 1 (TAK1) (Kim, et al., 2008), a serine kinase which activates Ik kinases (IkK) in complex with the TAK1 binding protein1 (Tab1) (Sato, et al., 2005). This activation of IkK then leads to the phosphorylating of IkB, the inhibitor of NF-kB. In addition, phosphorylation of IkK can also be achieved by binding of RICK to NEMO. This binding leads to ubiquitinylation of NEMO and the activation of its subunits Ikα and Ikkβ (Abbott, et al., 2004). These subunits phosphorylate IkB. After its phosphorylation, IkB is ubiquitinylated and degraded by the proteasome (Figure 2). For Nod1, the exact transduction of the signal is not clear yet. Degradation of IkB releases NF-kB which translocates into the nucleus and acts as a transcription factor (Elewaut, et al., 1999).

1.2.2 Regulation of IL-8 expression

IL-8 expression is regulated on many different levels and rather complex (Hoffmann, et al., 2002). This is not surprising since IL-8 is such a powerful neutrophil attracting chemokine (Baggiolini and Clark-Lewis, 1992). There are two binding sites for transcription factors in the IL-8 promotor regulating transcription. An NF-kB binding site is located between nucleo-

tides -1 to -133 within the 5' flanking region of the IL-8 gene, which is essential and sufficient for basal transcription (Mukaida, et al., 1994). However, maximal induction of gene expression requires binding of additional transcription factors such as activating protein 1 (AP-1) (Lee, et al., 1997) or CAAT/enhancer-binding protein (C/EBP) (Matsusaka, et al., 1993). These sites are dispensable for activation in some cells, but contribute to activation in others. How C/EBP binds to the IL-8 promotor is largely unknown. AP-1 binds the promoter as a homo- or heterodimer composed of c-JUN, JUN D, JUN B, ATF-2, c-FOS, Fra-1, FRA-2 and others (Karin, et al., 1997). Mitogen activated protein kinases (MAPK) regulate IL-8 expression via AP-1. Jun-N-terminal kinase (Jnk), p38 and the Extracellular regulated kinase (Erk) are involved in this process. Stimuli that activate NF-kB also activate Jnk and antisense mediate blockage of JNK leads to diminished IL-8 expression in human epithelial cells (Krause, et al., 1998).

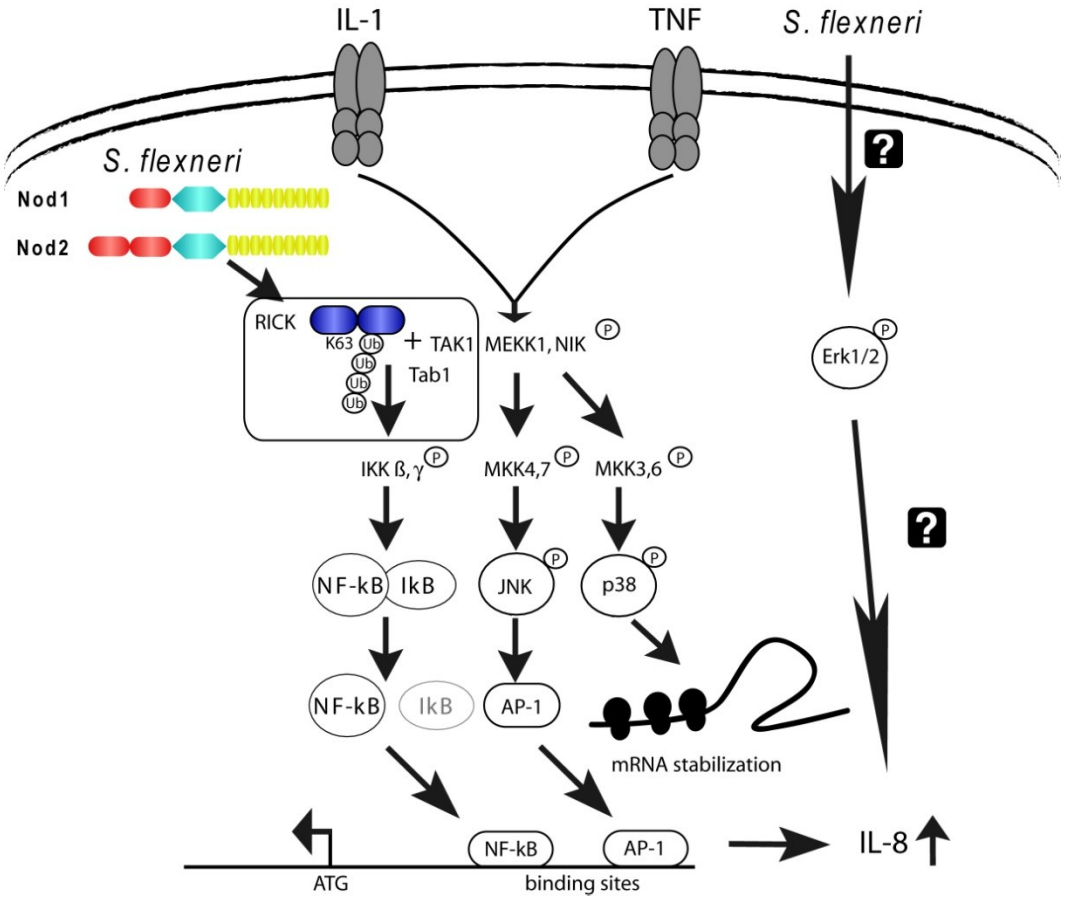

Figure 2: Regulation of IL-8 expression IL-8 expression is regulated by different pathways.

NF-kB activation is strictly required for IL-8 expression while MAPK activation increase protein levels even further. NF-kB activation is achieved by detection of peptidoglycan fragments by the intracellular pattern recognition receptors Nod1 and Nod2. They activate RICK which in turn forms an activation complex with TAK1 and Tab1. This activation complex then leads to the phosphorylation of the Ik kinases β and γ which in turn phosphorylated the inhibitor of kB (IkB). Degradation of IkB after its phosphorylation then allows NF-kB to translocate into the nucleus and act as a transcription factor. Stimuli leading to activation of MAPK by *Shigella* are largely unknown. CARD domains are depicted in red, NACHT domains in blue and LRR domains in yellow.

In addition, p38 stabilizes the IL-8 mRNA leading to even higher expression levels, but its activation is not required for transcription (Holtmann, et al., 2001). Erk – which has been reported to be activated during *Shigella*

infection of epithelial cells (Kohler, et al., 2002) – also contributes to the levels of IL-8 expression. The mechanism, however, is not clear. Interestingly, TAK1, which links Nod dependent detection of *Shigella* to NF-kB activation, is also involved in IL-1 or TNF-induced activation of MAPK (Shim, et al., 2005). Therefore, activation of Nod1 or Nod2 by *Shigella* can both induce NF-kB and MAPK activation required for maximal expression of IL-8 (Figure 2).

1.3 Neutrophils

Neutrophils (also called polymorphonuclear cells) are the most abundant white blood cell type. They make up to 70 percent of all leukocytes and show a characteristic multilobular nuclear shape and a granular cytoplasm (Wheater and Burkitt, 1987). Neutrophils develop in the bone marrow (Gallin and Snyderman, 1999). After leaving the bone marrow, neutrophils are terminally differentiated and short-lived cells (Murphy, et al., 2007). They circulate in the blood for about 24 hours before they enter apoptosis. Neutrophils are designed to detect, attack and kill infecting microorganisms. Because neutrophils leave the circulation and migrate towards an infection very fast, they are the first line of defense of the innate immune system against invading pathogens (Gallin and Snyderman, 1999). Diseases resulting in a reduced number of neutrophils in the blood, such as chronic granulomatous disease, are very often fatal because of recurrent life threatening bacterial and fungal infections of the patients (Dinauer and Orkin, 1992).

1.3.1 Antimicrobial mechanisms

Neutrophil antimicrobial mechanisms work either oxygen-dependent or oxygen-independent. Oxygen-dependent killing of microbes is characterized by the generation of Reactive Oxygen Species (ROS) by the NADPH

oxidase at the membrane of the phagolysosome. The NAPDH oxidase is activated by a drop in pH during phagolysosomal maturation and produces superoxide anions (O_2^-) (Babior, et al., 2002; Batot, et al., 1995). Superoxide anions are quickly converted into H_2O_2, which reacts with halide ions to yield hypohalides. These hypohalides are very potent antimicrobial molecules (Klebanoff, 2005). Oxygen-independent killing is achieved by antimicrobial proteins and peptides residing presynthesized in granules throughout the cytoplasm (Faurschou and Borregaard, 2003). These AMPs reach their target by three different mechanisms: phagocytosis, NET formation and degranulation depending on the distance between neutrophil and microbe as well as activation stimulus and activation time.

1.3.2 Phagocytosis

Neutrophils phagocytose microbes they encounter physically. They can phagocytose either the pathogen directly or the opsonized microbe (Gordon, 2002). Opsonization is binding of antibodies or complement to the surface of the microorganism, which enhances its recognition by phagocytes (Foster, 2005). Antibodies attached to their target can be bound by three different receptors (F_c-receptors) on neutrophils that recognize their constant region (F_c). In non-activated neutrophils, FcγRIIA (CD32) and FcγRIIIb (CD16), are the main receptors while the high-affinity receptor FcγRI is upregulated after induction with interferon (McKenzie and Schreiber, 1998). IgA class antibodies bind to the FcαR receptor (CD89) expressed by neutrophils. Bound complement is recognized via the β2 integrin MAC1 (CD18/CD11b or CR3). After its activation MAC1 binds to C3bi deployed on the particle to be phagocytosed. Other complement receptors are CR1 (CD35) and CR4 (CD11c/CD18) (Daeron, 1997; Gessner, et al., 1998; Petty and Todd, 1993). Complement and antibody mediated phagocytosis show differences with respect to the uptake mechanism. While complement covered particles seem to sink into the cell, antibody

tagged microbes are taken up by the fast formation of pseudopods which enclose the microbes (Greenberg and Grinstein, 2002). Phagocytosis by both mechanisms leads to the formation of a phagosome which subsequently maturates into the phagolysosome. Phagocytosed particles have to be larger than 0.5 µm in diameter. Therefore viruses cannot be phagocytosed. Formation of the phagosome includes a drop of the pH and fusion of the phagosome with granules. This fusion leads to the release of AMPs into the phagolysosome (Demaurex, et al., 1993; Hampton, et al., 1998). In addition, drop of the pH activates the NADPH oxidase which produces superoxide anions (O_2^-). Together, AMPs and production of ROS in the phagosome kill internalized microbes.

1.3.3 NET formation

Neutrophil extracellular traps (NETs) consist of extracellular chromatin, i.e. histones bound to DNA, which is decorated with granular proteins (Brinkmann, et al., 2004). These proteins include – among others - neutrophil elastase and myeloperoxidase. NETs build an extracellular mesh capable of capturing and killing gram-negative (Brinkmann, et al., 2004) and gram-positive bacteria (Fuchs, et al., 2007) as well as fungi (Urban, et al., 2006). NETs are generated after prolonged activation of neutrophils for example by activation of F_c receptors or stimulation of Protein kinase C (PKC) with PMA. Formation of NETs strictly requires NADPH oxidase activity. Neutrophils from patients with loss of function mutations in the NADPH oxidase are not able to make NETs (Fuchs, et al., 2007).

1.3.4 Granules and degranulation

The antimicrobial proteins present in the granules of neutrophils represent the oxygen-independent arm of neutrophil based host defense. Granules

are cytoplasmic vesicles surrounded by a phospholipid bilayer. Their name originates from the grain-like appearance in blood preparations due to their dense packing. Distributed in four different types of granules are many different AMPs (Faurschou and Borregaard, 2003). AMPs are often cationic since this enhances their ability to bind to the negatively charged membranes of microorganisms. In addition, most proteins and peptides are bound to the polyanionic glycosaminoglycan matrix of lymphocyte granules (Stevens, et al., 1987; Tantravahi, et al., 1986). Release from the matrix is required for some of the AMPs in order to be biologically active. In addition, some AMPs require processing either on their way to the granules or after release from the granules to form the biologically active cytotoxic molecule (Sorensen, et al., 2001). The four different types of granules in neutrophils are azurophilic (primary), specific (secondary), gelatinase (tertiary) granules and secretory vesicles (Faurschou and Borregaard, 2003). These granules arise at different time points during neutrophil development as immature transport vesicles which fuse after budding off the Golgi apparatus. Their contents are targeted to the granules simply by the time at which they are synthesized during development (Borregaard, et al., 1995; Le Cabec, et al., 1996). The distribution of granular proteins in the different granule types is listed in table 1. These proteins can either be directed to the phagosome or the extracellular space. Degranulation is the fusion of the granules with the plasmamembrane (exocytosis) which is triggered by inflammatory mediators such as the bacterial peptide fMLF (Sengelov, et al., 1993) or by ligation of L-selectin (Laudanna, et al., 1994) and CD11b/CD18 (Ng-Sikorski, et al., 1991) as well as by stimulation with PMA (Faurschou, et al., 2002). SNAREs are likely involved in the fusion process (Mollinedo, et al., 2006). Signaling leading to degranulation is transmitted via a rise in cytosolic Ca^{2+} levels and an alternative yet undefined pathway. The release of AMPs from the granules follows a defined order *in vitro* (Sengelov, et al., 1993)

as well as *in vivo* (Sengelov, et al., 1995): Secretory vesicles release their contents first, followed by gelatinase granules, specific granules and lastly azurophilic granules.

Table 1: Granular proteins of neutrophils Abbrevations:R, receptor; CRISP, cystein-rich secretory protein; SGP-28, specific granule protein of 28 kDa; uPA, urokinase-type plasminogen activator, adapted from (Faurschou and Borregaard, 2003)

Azurophil granules	Specific granules	Gelatinase granules	Secretory vesicles
Membrane CD63 CD68 Presenilin 1 Stomatin V-type H^+-ATPase fMLF-R Fibronectin-R G-protein$_\alpha$ subunit Laminin-R Leukolysin NB1 antigen 19-kDa protein 155 kDa protein Rap1, Rap2 SCAMP SNAP-23, -25 Stomatin Thrombospondin-R TNF-R uPA-R VAMP-2 Vitronectin-R	CD11b / CD18 CD15 CD66 CD67 Cytochrome b_{558} NRAMP-1 SCAMP SNAP-23, 25 uPA-R VAMP-2 V-type H^+-ATPase	CD11b / CD18 CD67 Gp91phox/p22phox MMP25 TNFR SNAP-23 Cytochrome b_{558} Diacylglycerol deacetylating enzyme fMLF-R Leukolysin CD16 CD45 CR1 C1q-R Cytochrome b_{558} Decay-accelerating factor (DAF) fMLF-R Leukolysin VAMP-2 V-type H^+-ATPase Arginase-1	Alkaline phosphatase CD10 CD11b/CD18 CD13 CD14 LIR1-4,-6,-7,9 CD35 CD16 C1q-R
Matrix Acid β-glycerophosphatase Acid mucopolysaccharide α_1-antitrypsin α-Mannosidase Azurocidin BPI β-Glycerophosphatase β-Glucuronidase Cathepsins Defensins Elastase Lysozyme MPO N-acetyl- β-glucosaminidase Proteinase-3 Sialidase Ubiquitin-protein	β_2-Microglobulin Collagenase CRISP-3 (SGP-28) hCAP18 Histaminase Lactoferrin Lysozyme NGAL uPA Sialidase Transcobalamin-I	Acetyltranferase β_2-Microglobulin CRISP-3 Gelatinase Lysozyme	Plasmaproteins

This order makes particular sense since it resembles the response of neutrophils during their approach to the site of infection. Secretory vesicles are easily triggered for degranulation (Sengelov, et al., 1993; Sengelov, et al., 1993). They contain mainly serum derived proteins and membrane located receptors needed for neutrophil transmigration through the vascular endothelium. Many of these proteins locate to the plasmamembrane of neutrophils after exocytosis. For example, the complement receptors CR3 (MAC-1) and CR but also receptors recognizing microbial pattern such as the fMLF-receptor and the TLR-4 co-receptor CD14 can be found in the membrane (Borregaard, et al., 1994). In addition, FcγIII receptor CD16 and the metalloprotease leukolysin are transferred to the plasmamembrane. L-selectin is shedded from the surface of neutrophils after their mobilization (Borregaard, et al., 1994). All these changes of localization enable the neutrophil to establish tight contact with the vascular endothelium (Faurschou and Borregaard, 2003). Release of other granule proteins such as neutrophil elastase at this point would provoke tissue damage at an unwanted site. Gelatinase granules are exocytosed next. These granules are low in antibiotic substances and are more easily released than specific granules. Gelatinase granules contain mainly matrix degrading enzymes needed for extravasation and diapedesis (Faurschou and Borregaard, 2003). Proteases include matrix metalloprotease-8, gelatinase and leukolysin. Antimicrobial proteins present in specific granules include hCAP18, the only known human cathelicidin (Cowland, et al., 1995). Following exocytosis, the c-terminal peptide LL-37 is released from hCAP18 by proteolysis through proteinase-3 (Sorensen, et al., 2001). LL-37 exhibits antimicrobial activity against gram-positive and gram-negative bacteria. It is an α-helix that binds to the Lipid A part of LPS (Kai-Larsen and Agerberth, 2008). Another antimicrobial protein present is lactoferrin which inhibits microbial growth by two different mechanisms. Lactoferrin is an iron-sequesting glycoprotein, causing impairment of bacterial growth

(Oram and Reiter, 1968). In addition, an amphipatic α-helical part of lactoferrin inserts into bacterial membranes of both gram-negative and gram-positive bacteria (Chapple, et al., 1998). The neutrophil gelatinase-associated lipocalin is also stored in specific granules and exists as monomers and homodimers (Kjeldsen, et al., 1994; Kjeldsen, et al., 1993). It is bacteriostatic by binding ferric siderophores (Goetz, et al., 2002). Lysozyme is a granular protein present in all granule types but with the highest concentration in specific granules. It cleaves peptidoglycan polymers of the bacterial cell wall (Selsted and Martinez, 1978). In azurophilic granules, mostly antimicrobial proteins and peptides can be found. Azurophilic granule exocytosis takes place last during degranulation. Many proteins in these granules have to be processed in order to be active. Azurophilic granules also contain the hypohalide generating enzyme myeloperoxidase. In addition the serprocidins - serine proteases with bactericidal activity - neutrophil elastase, cathepsin G and proteinase-3 are stored. These proteases degrade many extracellular matrix proteins such as fibronectin, elastin, laminin, type IV collagen and vitronectin. Therefore they are essential for extracellular matrix degradation during diapedesis. Azurophilic granules contain another set of antimicrobial peptides called α-defensins. These are small cationic peptides (about 3.5 kDa), abundant in azurophlic granules and are made during the promyelocyte to myelocyte stage. They make up to 5% of the protein content of neutrophils and are bactericidal against gram-positive and gram-negative bacteria (Ganz, et al., 1985). Defensins are synthesized as pro-forms that are cleaved by azurophilic granule proteases. Bactericidal / Permeability Increasing Protein (BPI) is a 50 kDa highly cationic antimicrobial protein, which kills gram-negative bacteria at nanomolar concentrations (Elsbach, 1998; Weiss and Olsson, 1987). Between 0.5 to 1 % of neutrophil protein content can be attributed to BPI. BPI binds to negative charges of the LPS of gram-negative bacteria. Positive charges defining its cationic property are concentrated in a

discrete region at the N-terminus of this linear molecule (Beamer, et al., 1997). Binding to LPS induces rearrangements in the bacterial membrane lipids and leads to growth inhibition at lower concentrations followed by bactericidal activity through damage at the inner membrane (Mannion, et al., 1989; Mannion, et al., 1990). The C-terminal domain of BPI has an opsonizing function which mediates bacterial attachment to neutrophils and monocytes resulting in phagocytosis (Iovine, et al., 1997).

1.4 Lipopolysaccharide

1.4.1 Structure of LPS

Lipopolysaccharide is a major constituent of the outer membrane of gram-negative bacteria. Together with phospholipids, proteins, lipoproteins and other conponents, it is responsible for the structural integrity of the bacterial envelope (Rietschel, et al., 1996). Because of its importance, it is highly conserved and indispensible. Therefore, the immune system of multicellular organisms can detect gram-negative bacteria via recognition of LPS (Sultzer, 1968). LPS is also known as endotoxin, since it elicits very strong proinflammatory responses in infected host that lead to septic shock and eventually death. The LPS of gram-negative bacteria shares a common structural organization. It consists of a Lipid A, an inner and outer core region and the O-antigen (Figure 3).

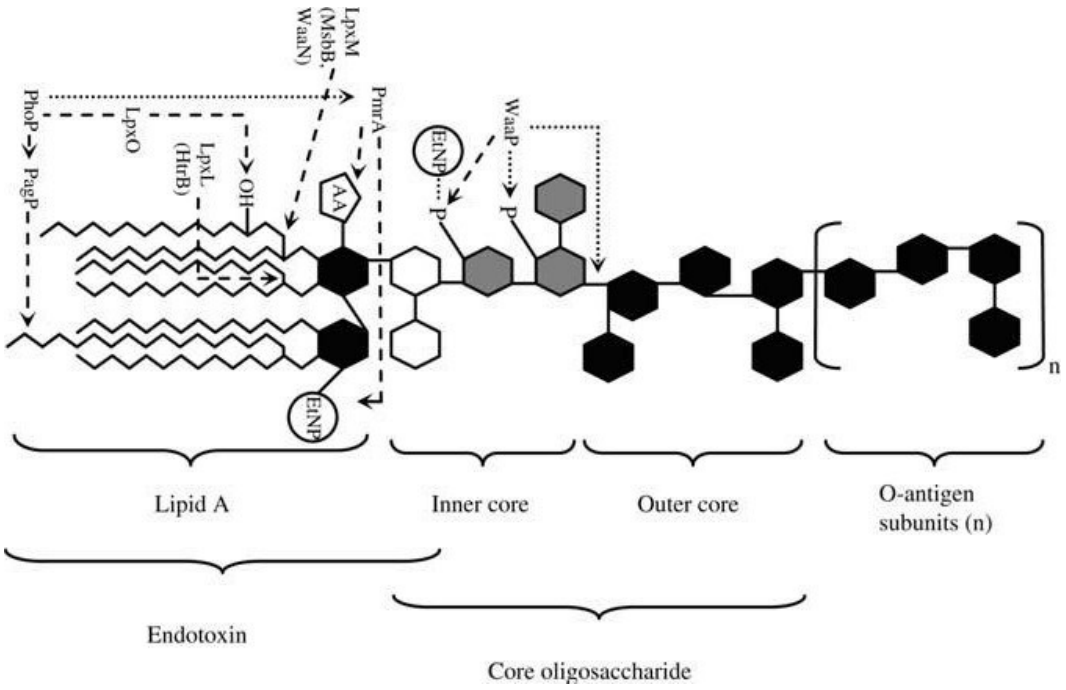

Figure 3: The Structure of Lipopolysaccharide

Depicted is the general structure of LPS (from *S. enterica* serovar Typhimurium). LPS consists of LipidA, the inner and outer core as well as the O-antigen. The Lipid A carries 2 hexaacylated glucosamine residues. The inner core is made of two Deoxy-D-manno-oct-2-ulosonic acid residues and two following heptoses. The outer core carries glucose, galactose which is branching to two glucose molecules and then N-acetyl-glucosamine.In *Shigella* there are slight differences to this general structure. For example, modifications by aminoarabinose (AA) or Ethanolamine (EtNP) are not present. Other differences are described below. (Taken from (Nagy and Pal, 2008))

1.4.1.1 Lipid A

The structure of Lipid A is very similar among enterobacteria. Lipid A consist of a β-D-glucosaminyl-(1-6)-α-D-glucosamine disaccharide, which is phosphorylated at the O-1 position of the reducing and at the O-6 position of the non-reducing glucosamine residue. In addition, the two glucosamine residues are hexaacylated by four directly linked acyl chains of which two are itself acylated (Figure 3). The acyl chains of S. *flexneri* and *Escherichia coli* consist of tetradecanoic and dodecanoic fatty acids. Fatty acids

directly linked to the N-acetyl-glucosamine residues are hydroxylated at the C-3 position (Lindberg, et al., 1991). This structure consisting of the acylated and phosphorylated glucosamine disaccharide is functionally sufficient to elicit all observed symptoms during endotoxic shock (Rietschel, et al., 1996).

1.4.1.2 Core region

Shigella flexneri M90T used in this study is of the serotype 5a. The structure of the O-antigen defines the serotype of a specific strain. The core region of *Shigella flexneri* serotype 5a LPS consists of two molecules of 3-Deoxy-D-manno-oct-2-ulosonic acid linked to Lipid A via the O-6 position of the nonreducing glucosamine followed by two phosphorylated heptose molecules. The first heptose carries a phosphoethanolamine group. However, this modification has only been confirmed for other serotypes and not 5a (M90T) and might differ. The heptoses are followed by glucose, galactose which is branching to two glucose molecules and then N-acetyl-glucosamine.

1.4.1.3 O-Antigen

The structure of the O-antigen of *Shigella flexneri* serotype 5a is defined in detail (Figure 4). It consists of pentameric repeats of N-acetylglucosamine and rhamnose residues with cross branched glucose residue at the second rhamnose (Kenne, et al., 1977; Kenne, et al., 1978).

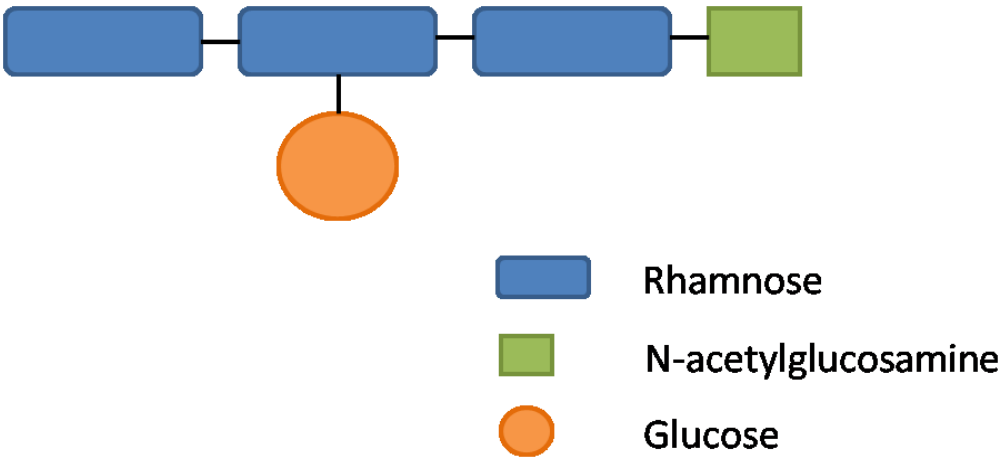

Figure 4: Structure of the *Shigella flexneri* serotype 5a (M90T) O-antigen

The *Shigella flexneri* serotype 5a (M90T) O-antigen consists of a pentameric repeat of three rhamnose residues with a cross branched glucose residue at the second rhamnose followed by one N-acetylglucosamine residue (adapted from (West, et al., 2005)).

The number of O-antigen repeats is not fixed. In *Shigella* two modes of O-antigen length exist which are regulated by the Cld protein. Mode A consists of about 15 and Mode B of 100 O-antigen repeats (West, et al., 2005).

1.4.2 LPS and virulence

Since LPS is a surface molecule, it interacts with the host during infection by gram-negative pathogens. Its exposure together with its indispensability makes it an excellent target for antimicrobial defense molecules of the host. Bacteria have countered this weakness by modifying their LPS structure in order to be less susceptible against AMPs. For example, *Salmonella typhymurium* serovar *typhi* modifies its LPS regulated by the stress induced *pmrA/B* regulon (Gunn, et al., 1998). Activation of this operon results in addition of phosphoethoanolamine and aminoarabinose to core sugars or phosphate groups of Lipid A (Figure 3), rendering these moieties less negatively charged. Other bacteria such as *Helicobacter pylori,* Lep-

tospira *interrogans* or *Francisella novicida* either remove or methylate these phosphate groups, leading to a modified TLR4 recognition of the modified LPS (Boon Hinckley, et al., 2005; Tran, et al., 2004; Wang, et al., 2004). Since these charges often mediate binding of AMPs to Lipid A and core residues, these modifications improve the resistance of the bacteria against AMPs. In the case of Salmonella, modification of its LPS reduces binding of polymyxin, an antimicrobial peptide (Vaara, et al., 1979). Furthermore, the chain length of LPS influences binding of BPI to bacteria (Capodici, et al., 1994). Besides its structural role, LPS can serve as an adhesion during infection. In guinea pig, addition of isogenic *Shigella* LPS inhibits adhesion to colonic epithelial cells (Izhar, et al., 1982).

1.5 Aim of the study

Neutrophils are the first cells of the innate immune system recruited to the site of *Shigella* infections. Degranulation starts upon detection of bacterial molecules by neutrophils. This releases many antimicrobial proteins and peptides as well as enzymes from the granules into the extracellular space which are bacteriostatic, bactericidal or degrade virulence factors. Several of these granular proteins have been characterized with respect to their direct effect on the bacterium. However, it is currently not known how sub-lethal concentrations of these proteins released by degranulation affect *Shigella* virulence. One major virulence feature of *Shigella* is the invasion of epithelial cells by induction of its phagocytosis where it is protected from attacks of the immune system. In this study, we analyzed the effect of granular protein binding to *Shigella* on its adhesion and invasion of epithelial cells. We show that binding of neutrophil antimicrobial proteins strongly enhances *Shigella* adhesion to and invasion in epithelial cells. This “hyper-invasion” causes massive infection of epithelial cells in the presence of degranulating neutrophils. In addition, we show that hyperinvasion is mediated by electrostatic interactions and requires binding of cationic proteins to the *Shigella* surface. This binding also depends on the structure and charge of the *Shigella* LPS. We propose that *Shigella* evolved to use host defense molecules to enhance its virulence and subvert the innate immune system.

2 Materials and Methods

2.1 Chemicals

All chemicals were obtained from Sigma unless otherwise stated.

2.2 Media

Tryptic Soy Broth (TSB)

30 g Tryptic Soy Broth
/ 1 l H2O

Luria-Bertani media (LB)

10 g Bacto tryptone
5 g Bacto yeast extract
10 g NaCl
/ 1 l H2O

Adjust to pH 7.0 with 5N NaOH

2.3 Buffers / Reagents

TBS-T (Tris buffered saline – Tween 20)

200 mM NaCl

50 mM Tris-HCl pH 7.4

0.05 % Tween 20

TAE (Tris/Acetic acid/EDTA) (50X)

242 g Tris-Base

57.1 ml Acetic acid

100ml 0.5M EDTA

Add ddH2O to 1 liter and adjust pH to 8.5.

Agarose gel-loading buffer (6x)

0.25% (w/v) Bromphenol blue,

0.25% (w/v) Xylene cyanol FF

30% (v/v) Glycerol in H2O

Comassie staining solution

50 % Methanol

10 % Acetic acid

0.05% Brilliant blue (R-250)

Comassie destaining solution

50 % Ethanol

10 % Acetic acid

2.4 Strains and cell culture

2.4.1 Bacterial strains

Shigellae were grown on TBA plates including 0.01% Congo red. *Shigella*'s ability to bind Congo red correlates with the presence of the virulence plasmid (Qadri, et al., 1988). The wild-type strain M90T that harbours the virulence plasmid pWR100 was used for virulent infections (Sansonetti, et al., 1982). The avirulent BS176 strain served to prove for any effects not derived from specific virulence factors of *Shigella* (Sansonetti, et al., 1982). For overnight cultures, a single colony from a plate was grown in 5 mL TSB-medium at 37°C shaking at 200 rpm. Overnight cultures were subcultured 1:100 in TSB. *Shigella* LPS mutants *ΔrfbA, ΔgtrA, ΔgtraB, Δcld, ΔwaaD, ΔwaaJ* and *ΔwaaL* were kindly provided by Christoph Tang (Imperial College) and their generation is mentioned elsewhere (West, et al., 2005). *Shigellea* were grown in Tryptic Soy Broth and LB agar (Difco).

2.4.2 Cell culture

Hela cells were obtained from the ATCC and propagated in DMEM containing 10 % FBS, Penicillin / Streptomycin and L-Glutamamine. Cells were passaged routinely every 2-3 days when reaching 80% confluence by trypsination and dilution with fresh medium. One day before infection, cells were seeded in antibiotic free complete medium. RPMI, DMEM, HEPES, PBS w/o calcium, HBSS w/o calcium, L-Glutamine and pyrogen free ultra pure water were obtained from Gibco. FBS and Trypsin / EDTA were purchased from Biochrom AG.

2.5 Biochemical methods

2.5.1 SDS PAGE gel electrophoresis

Proteins were separated using reducing sodiumdodecylsulfate polyacrylamide gel electrophoresis. Gels were obtained precast from Biorad. These gels were Bis-Tris buffered gradient gels from 4-12%. MOPS running buffer was used (Biorad) and samples were prepared in XT sample buffer (Biorad) with reducing agent added (Biorad) by incubation at 95°C for 5-10 minutes. Electrophoresis was carried out at 120 V for 2 hours at room temperature. For analysis of LL-37 binding to *Shigella* a 16.5 % Tris-Tricine gel from Biorad was used.

2.5.2 Western blot

After electrophoresis, proteins were transferred onto polyvinylidenfluorid (PVDF) membranes. The membrane was activated by incubation in methanol. For transfer, the membrane was put onto the gel and both were surrounded by prewetted filter paper. All components were presoaked in transfer buffer for 5 minutes.Transfer was done at 350 mA for 45 minutes at 4°C.

2.5.3 Enzyme-linked immunosorbent Assay (ELISA)

Enzyme-linked immunosorbent assays (ELISAs) allow the quantification of proteins based on specific antibody binding. Sandwich ELISAs rely on protein capture with an immobilized antibody and subsequent detection with an antibody detecting a different epitope. This second antibody is labelled, e.g. with biotin, which allows subsequent detection with streptavidin-HRP and a quantitative TMB substrate reaction. Cytokines in cell culture supernatants were quantified by ‘sandwich’ ELISA according to the manufaturer’s instruction (R&D Systems).

2.5.4 Protein concentration determination

Protein concentration was determined using a BCA assay (Pierce) as described by the supplier. Absorbance was measured on a SpectraMax 190 microplate reader.

2.6 Molecular biology

2.6.1 Primers

Primers used were supplied by MWG and resuspended at 100 µM. The sequences were as follows

K2 CGGTGCCCTGAATGAACTGC

K1 GCCGATTGTCTGTTGTGCCC

Kt CGGCCACAGTCGATGAATCC

BipA 5'-test GTGATCCTGTTTTGTAGTGC

BipA 3'-test AACGAAATTAATCGTCTTTC

IpaH9.8 5'-test AACTTCATAAAAACTCCTAC

IpaH9.8 3'-test GTACGTATTGACAGGTTTCA

BipA-3'+P4:
TCTTTCGGTGCGCGGTTGGCGCGGCGACGATCGTTTTCCGATTCCGG
GGATCCGTCGACC

IpaH9.8-3'+P4:
CCTGTCCTATCACTGGCGCTGACAGTTTTATGCGATGTGAATTCCGGG
GATCCGTCGACC

IpaH9.8-5p+P4:
ACAAAGCCATTTGTCCACCGGCTTTAACTGGATGCCCATCGTGTAGGC
TGGAGCTGCTTC

2.6.2 Polymerase Chain Reaction

PCR was performed using either PCR ready mix (Invitrogen), gentherm polymerase (Rapidozym) or native Pfu polymerase (Stratagene). Nucleotides were from Rapidozym. For generation of knock-out cassettes, the pKD13 or pKD4 vectors served as templates. Primers consisted of a 20 nucleotide region annealing to the kanamycin cassette and a 30 nucleotide long extension homologous to the region to be deleted. The knock-out cassettes were generated using the following program on a stratagene robocycler:

1µl Gentherm polymerase

1µl dNTP

1µl 5p Primer (20µM)

1µl 3p Primer (20µM)

1µl Template pKD13 (miniprep 20 ng/µl)

5 µl Gentherm buffer w/ Mg

40 µl ddH2O

Program:

94°C	3´		
94°C	45´´	-------	|
50°C	45´´		| 30x
74°C	2´	-------	|
74°C	7´		

For confirmation of knock-outs, primers were designed inside the kanamycin cassette as well as about 100 bp inside the gene of interest. Three different combinations were used to confirm the knock-out.

2.6.3 PCR product purification

After amplification, the whole PCR product was mixed with 6x DNA loading buffer and loaded on a 1 % agarose gel. The gel was run at 80 mA for 30 - 45 minutes. The band corresponding to the product was cut out on an UV-table, DNA was purified using Qiagen gel extraction kit and eluted either in 30 or 50 µl of ddH2O depending on the amount of PCR product as examined by the agarose gel. For purification of the kanamycin cassette, three PCR reactions were combined to concentrate the product.

2.6.4 Agarose gel electrophoresis and gel extraction

Routine agarose gel electrophoresis was done using 1% agarose gels in 1 x TAE. Gels were run at 80-100 V for 30-60 minutes. As marker, Smartladder from EuroGentech was used.

2.6.5 Isolation of plasmid DNA

For plasmid DNA preparations isolation kits from Qiagen were used according to the manufacturer's instructions. Maxi-Preps were done endotoxin free.

2.6.6 Gene Knock-out in *Shigella*

Genes were knocked out by using the method of Datsenko-Wanner (Datsenko and Wanner, 2000). PCR products containing the kanamycin cassette were prepared using primers as described above.

2.6.7 Preparation of electrocompetent *Shigella* and *E. coli*

Electrocompetent bacteria were prepared by inoculating E.*coli* DH5α or *Shigella flexneri* M90T in 2 ml LB media over night at 37 °C with shaking. The next day, bacteria were subcultured 1:100 in 100 ml of LB and incubated at 37°C for 3 hours with shaking. Then, bacteria were put on ice for 15 minutes and pelleted by centrifuging for 15 minutes at 6000 g at 4 °C. Supernatant was removed and bacteria were washed twice with ice cold sterile ddH_2O. The bacteria were resuspended in 1 ml of 10 % glycerol in H_2O and transferred to an Eppendorf tube. Bacteria were pelleted by centrifuging at top speed in a cooled microcentrifuge at 4°C for 1 minute and resuspended in 400 µl of 10 % glycerol in H_2O. These were aliquoted into 45 µl aliquots, snap frozen in liquid N_2 and stored at –80°C. For transformations to generate knock-outs, electrocompetent cells were prepared

freshly each time and directly used for transformation of the kanamycin cassette.

2.6.8 Transformation

For knockouts using the Datsenko-Wanner method, 200 ng – 2 µg of PCR product were were used to transform 45 µl of electrocompetent M90T in an electroporation cuvette (0.1 cm). Bubbles were removed and cells were electroporated at a voltage of 1.8 kV (E = 18 kV/cm) with time constants reaching from 2 to 5 ms. After electroporation, bacteria were taken up in 500 µl TSB media and incubated at 37°C for 1 hour in a shaker. The total transformation mix was plated on TSB-Agar plates and incubated at 37°C over night.

2.7 Transfection of HeLa cells

HeLa cells were transfected by preparing the tranfection mix described in table 2. Serum free media was used for the preparation. Four hours after transfection, cells were washed and fresh media containing serum was put. Analysis of transfected cells was done at 24 hours after transfection.

Table 2: Transfection of HeLa cells for NF-kB reporter assay

	16x renilla / firefly	4x GFP	4x renilla
Total Volume	512 (2x)	128 (2x)	128 (2x)
Lipofectamin 2000	20 µl	5	5
pGEM-NF-kB-luc	7200 ng	-	-
pRL-TK	800 ng	-	2000
pEF1:IkB-GFP		2000 ng	-

2.8 NF-kB activation assay

To measure activation of NF-kB in infected cells, HeLa cells were transfected with vectors encoding firefly luciferase as well as renilla luciferase. The firefly luciferase is under control of a NF-kB inducible promoter while the renilla luciferase is consitutively expressed. This consistutive expression is used to normalize for different well-to-well transfection efficiencies. The firefly expressing vector used was the pGEM-NF-kB-luc. The NF-kB recognition site was from the angiotensin-promoter and had the following sequence: GATCCACAGTTGGGATTTCCCAACCTGACCAGA
The vector encoding for consitutively expressed renilla luciferase was the pRL-TK vector. For the assay, cells were seeded at $3*10^4$ per well in 48-well-plate in antibiotic free media to be transfected the next day. This gave 75-85% confluent cells at the day of transfection and a monolayer the day after used for infection. 100 ng of pGEM3 and 10 ng of pRL-TK vector were used per transfection. Cells were also transfected with pEF1:IkB-GFP to check for transfection effiency microscopically. For TNF-α pre-treatment, cells were incubated for 30 minutes with 20 ng/ml TNF-α (R&D). Three hours after infection with hNGP pre-treated *Shigella*, media was taken off and cells were lysed for 10 minutes on a shaker in 75 µl of passive lysis buffer / well. 30 µl of the lysate were put into a white 96-well luminometer plate and 50 µl of firefly-substrate was added (Dual-Glo, Promega). After 10 minutes, the plate was read. For analysis, light emission of renilla luciferase was used to normalize firefly luciferase relative light units (RLU).

2.9 Isolation of neutrophils

2.9.1 Dextran / Ficoll

Neutrophils were obtained from buffy coats (German Red Cross) or flushing back leukocyte reduction filters (Meyer, et al., 2005). Blood or elution buffer was then mixed with dextran (MP Biochemicals) at 1% final concentration and let stand for 30 minutes to allow for separation. Afterwards, the leukocyte enriched phase was overlaid on Ficoll-Paque Plus (GE Healthcare) and centrifuged for 30 minutes at 400 x g. Neutrophils and remaining erythrocytes are found in the pellet after centrifugation. Neutrophils were washed once with $HBSS^-$ at 250 x g for 10 minutes. Erythrocytes were lysed by addition of ice cold pyrogen free water for 30 seconds. Then RPMI / 10 mM HEPES was added to prevent lysis of neutrophils. After centrifugation at 250 x g for 10 minutes, neutrophils were taken up in RPMI / 10 mM Hepes and counted.

2.9.2 Histopaque / Percoll

First, Percoll solutions for the discontinous percoll gradient were prepared as follows:
Mix 36 ml Percoll with 4 ml 10x PBS (100 % isotonic percoll solution)
Mix 1.5 ml RPMI with 8.5 ml Percoll (85%)
Mix 2 ml RPMI with 8 ml Percoll (80%)
Mix 2.5 ml RPMI with 7.5 ml Percoll (75%)
Mix 3 ml RPMI with 7 ml Percoll (70%)
Mix 3.5 ml RPMI with 6.5 ml Percoll (65%)

2 ml of each solution were carefully layered with a plastic Pasteur pipette in a 15 ml falcon tube starting with 85% solution. Then, blood was collected into Vacutainer (K2E 10 mg / purple) by arm vein puncture. Five ml

collected blood was layered onto 5 ml of Histopaque 1119 in a 15 ml Falcon tube. The blood was centrifuged for 20 minutes at 800 x g at room temperature. After centrifugation, the interphase was discarded and the diffuse red phase of histopaque 1119 above the red blood cell pellet was collected. 5 ml of cells were washed with 10 ml of PBS containing 0.5% Human serum albuman (HSA) (Griffols) in a new centrifugation tube. Cells were centrifuged for 10 min at 300 x g at room temperature. Supernatant was removed and cell pellet resuspended in 2 ml PBS (0.5% HSA). This suspension was loaded on top of the Percoll (GE Healthcare) gradient. The gradient was then centrifuged for 20 minutes at 800 x g RT. The distinct white layer between clear 70% and 75% Percoll layers was collected in a 15 ml falcon tube. The tubes were filled up to 15 ml with PBS/HSA (0.5%) and centrifuged for 10 minutes at 300 x g RT. The cell pellet was then resuspended in 1 ml PBS/HSA (0.5%) and cells were counted using a Neubauer chamber.

2.10 Human Neutrophil Granular Proteins (hNGP) preparation

For preparation of hNGP 7 x 10^8 neutrophils corresponding to a packed volume of about 0.5 ml in a 15 ml falcon tube were used. 1.1 ml chilled pyrogen free water was added and neutrophils were sonicated for 2 x 30 seconds using a Bandelin Sono-plus sonicator equipped with a HD2070 sonication tip set to 25% power to lyse the cells. Lysis is complete when no cells sediment in the tube and the solution is milky white. Then H_2SO_4 is added to a final concentration of 0.16 M to the homogenized cells. The homogenate was then kept on ice and vortexed every 5 minutes for 30 minutes. From this step on all steps are performed in siliconized tubes since many of the cationic proteins stick to normal tubes. The acidic extract was centrifuged at 4°C for 30 minutes at 16000 x g. Supernatant was taken off and dialyzed against 2 liter of 20mM sodium acetate buffer (pH4) for 3 days with two exchanges in 3.5 kDa cutoff dialysis cassettes (Pierce).

After dialysis, the extract was again centrifuged at 4°C for 5 minutes at 16000 x g. hNGP is the supernatant of the second centrifugation step. hNPG was kept at 4°C and is stable for one month.

2.11 Invasion assay

2.11.1 hNGP treatment

Mid-log phase *Shigella* were resuspended in 1ml of PBS at a density of 1 x 10^8 containing hNGP or 20 mM sodium acetate buffer, pH4. hNGP was added last and bacteria were treated for 15 minutes at 37°C with slow shaking (80 rpm). After treatment, bacteria were centrifuged at 3300 x g for 5 minutes and resuspended in 1 ml PBS.

2.11.2 Poly-lysine / poly-arginine treatment

For treatment of *Shigella* with poly-lysine and poly-arginine respectively, bacteria were resuspended in 1 ml PBS containing the indicated concentration of poly-lysine or poly-arginine. Bacteria were incubated for 15 minutes at 37°C with slow shaking (80 rpm). After incubation, bacteria were centrifuged at 3300 x g for 5 minutes and resuspended in 1 ml PBS.

2.11.3 Gentamycin protection assay

Invasion of *Shigella* into epithelial cells was determined by performing a Gentamycin protection assay. *Shigella* were grown to mid-log phase (0.4 - 0.8) and treated with hNGP before infection. HeLa cells were seeded at 1 x 10^5 in 24-well-plates or 5 x 10^4 in 48-well-plates one day before the infection. Media on HeLa cells was exchanged to DMEM containing 2% FBS before infection. Cells were infected with *Shigella* resuspended in PBS at an MOI of 100. *Shigella* were centrifuged onto the cells by centrifugation at 300 x g, RT for 10 minutes. Infected cells were incubated for 30 minutes at 37°C and 5% CO2. Cells were washed twice with PBS and media contain-

ing 100 µg/ml Gentamycin was added. For analysis of invasion, cells were lysed 1 hour after Gentamycin treatment with 1% Triton (Roche) / PBS for 5 minutes with shaking. After lysis, intracellular colony forming units (CFU) were determined by plating of appropriate dilutions in liquid LB agar. For invasion assays using BPI and LL-37, a recombinant 25 kDa N-terminal fragment of BPI that has at least the same anti-microbial and LPS-neutralizing activities as does holo-BPI (Meszaros, et al., 1993) (kindly provided by Dr Jerrold Weiss, University of Iowa College of Medicine) and purified LL-37 (kindly provided by Dr. Mona Stahle-Backdahl Karolinska Hospital, Stockholm, Sweden) were used.

2.11.4 $MgCl_2$ elution

$MgCl_2$ elution was done by resuspending the bacteria in 200 mM $MgCl_2$ after hNGP treatment and incubation for 15 minutes at 37°C. After incubation, bacteria were centrifuged at 3300 x g for 5 minutes and resuspended in 1 ml PBS.

2.12 Adhesion assay

Treatment of bacteria and infection of cells was performed as mentioned, but directly after centrifugation of the bacteria onto the cells, cells were washed three times with PBS. Adherent bacteria were plated after treatment of the cells with 1%Triton/PBS.

2.13 Transwell system

HeLa cells were seeded in the lower chamber of a 0.4µm pore polycarbonate membrane transwell system (Corning) the day before the infection at 1 x 10^5 / well. The next day, neutrophils were purified using Histopaque / Percoll as described above. During purification of the neutrophils, the transwell inserts were saturated with protein by putting media containing

10% FBS for at least one hour. After purification, media was taken out of the insert and neutrophils were seeded in the insert in RPMI without phenol red containing 10 mM HEPES. Neutrophils were stimulated for 1 hour at 37°C in the CO_2 incubator using 1µM fMLF. During purification and stimulation, M90T were subcultured from the overnight culture 1:100 in TSB for 2-2.5 hours. After stimulation, the inserts containing the neutrophils were taken out and *Shigella*e were added to the lower chamber to be incubated with secreted neutrophil granular proteins for 30 minutes. After incubation, a gentamycin protection assay was performed as described above plating the invaded bacteria one hour after gentamycin treatment.

2.14 Hydrophobicity

2.14.1 Hydrophobicity interaction chromatography

Hydrophobicity interaction chromatography determines hydrophobicity by measuring the interaction of bacteria with either sepharose or a hydrophobic derrivative such as Octyl Sepharose CL-4B (Figure 24). Hydrophobic interactions are favored in the presence of higher concentrations of neutral salts. Depending on the salt concentration, hydrophobic molecules or patches on surfaces bind to the hydrophobic ligand present on the polymer matrix. Adhesion to the column containing sepharose defines background binding. Hydrophobicity is defined as percent retention of the bacteria to the octyl-sepharose column after background correction. The retention is measured as the OD of the bacteria before and after passage of the column. The choice of the sepharose modification as well as the type of salt and the salt concentrations tested affect the result of the experiment. We measured hydrophobicity after treatment of *Shigella* with hNGP (5%) or no treatment with hNGP. For every sample, background binding to the sepharose column was substracted and precent retention was calcu-

lated by dividing the OD of the flow-through by the OD of the input.

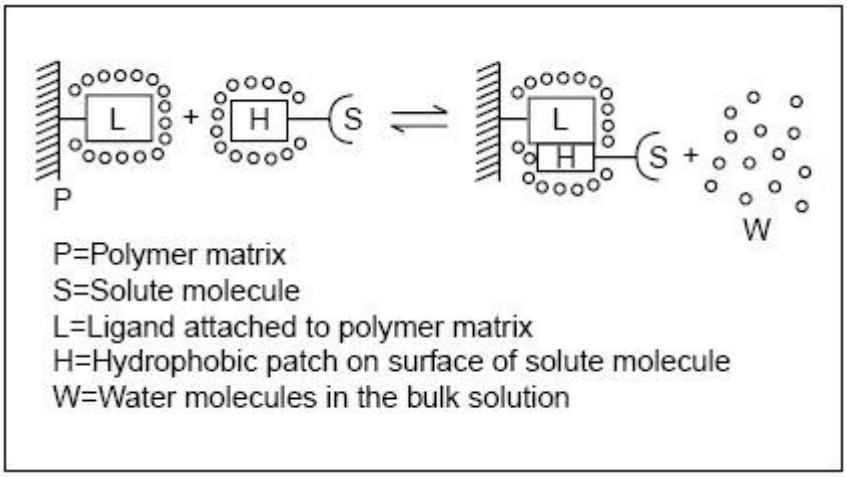

Figure 5: Principle of Hydrophobicity interaction chromatography

2.14.2 Microbial adhesion to hydrocarbon

Adhesion of microbes to organic solvents is measured using a MATH assay. The more hydrophobic a microbe, the better it binds to the organic solvent used. To measure adhesion, the bacteria are resuspended in PBS and overlayed with an organic solvent such as hexane. Then, the two-phase system is vortexed to allow the interaction of the bacteria present in the water phase with the organic solvent. The absorbance of the bacteria is measured before and after vortexing to determine the percentage of adherence to the organic phase.

2.15 Cytotoxicity / apoptosis assays

2.15.1 LDH release assay

Cytotoxicity of infected cells was determined using the Lactatedehydrogenase (LDH) assay from Promega. Cells were seeded at 5 x 10^4 per well the day before infection. Infection was performed as described for the gentamycin protection assay. After the indicated timepoints, supernatants (50µl) of infected cells were incubated with 50 µl of the substrate solution

until the positive controls showed good red dye development (5-15 minutes). The reaction was stopped using 100 µl of the provided stop solution. Absorption was measured at 450 nm on a SpectraMax 190 microplate reader. Cytotoxicity was defined as the percentage of LDH release from infected cells minuse uninfected controls divided by total LDH minus LDH release from control cells, using the following formula:

$$\frac{OD_{450}(sample) - OD_{450}(control)}{OD_{450}(total) - OD_{450}(control)} \bullet 100 = cytotoxici\ ty$$

2.15.2 Sytox assay

To measure cell death, HeLa cells were seeded in a 96-well-plate one day before the experiment in complete media without antibiotics. An overnight culture of M90T was setup and subcultured for 2 hours 1:100 in TSB. Media on cells was exchanged to serum free media one hour before infection. hNGP and poly-lysine treatment was done as described for the gentamycin protection assay. Cells were infected and plates were centrifuged at 300 x g for 10 minutes at RT to synchronize the infection. After 30 minutes, cells were washed twice with PBS and media containing gentamycin at 100 µg/ml was put including Sytox at a 1:1000 dilution. For total cell death, cells were permeabilized by putting 2 µl of 5% Saponin per well. Sytox fluorescence was measured every ten minutes using a 485 ex / 518 nm em filter pair in a ThermoScientific Fluorskan fluorescence reader incubated at 37°C with 5% CO_2.

2.15.3 TUNEL assay

$1x10^5$ HeLa cells were seeded on cover slips 24 hours before infection. Samples were prepared at 1 and 3 hours after infection by fixing the cells using 3.7 % Paraformaldehyde for 30 minutes at 4 °C. Permeabilization was done by immersing the slides in 0.2 % Triton X-100 in PBS for 5 mi-

nutes at RT and washing with PBS for 5 minutes twice. The positive control was set up by adding 100 µl of DNAse I buffer and incubation for 5 minutes at RT. Liquid was replaced by 100 µl DNAse buffer containing 10 unit / ml of DNAse I and cells were incubated for 10 minutes at RT. Slides were washed for 3 times with deionized water in a coplin jar separate from the other samples. Slides were layered with 100 µl equilibration buffer and equilibrated for 7 minutes. During that time, the TUNEL reaction mix was prepared by adding 5 µl labelled nucleotides and 1 µl of TdT-enzyme to 50 µl of Equilibration buffer. For the negative control, the TdT enzyme was substituted by ddH_2O. The reaction mix was added and cells were covered with provided plastic cover slips. Incubation was done in a humidified chamber at 37 °C in dark for 60 minutes. Reaction was stopped by immersing the slides in 2 x SSC for 15 minutes at RT. Samples were rinsed with PBS three times for 5 minutes at RT and mounted in Vectashield containing DAPI and analyzed by fluorescence microscopy.

2.16 Statistical analysis

Significance was tested using the students t-test or one-way Anova wherever apropiate. Calculations were done using the GraphPad 5.0 software. Differences were considered significant at a p-value < 0.05.

3 Results

3.1 Hyperinvasion

3.1.1 Human neutrophil granular proteins (hNGP) enhance *Shigella* adhesion and invasion

Granular proteins are released during degranulation of neutrophils into the extracellular space where they meet the bacterial surface. To test how neutrophil granular proteins affect *Shigella* adhesion and invasion, we generated a granule acid extract of purified human neutrophils (human neutrophil granular proteins (hNGP)) according to (Weiss, et al., 1978) with slight modifications to the protocol. We first determined sub-lethal concentrations of hNGP, i.e. concentrations at which at least 10 % of the bacteria survive. Treating *Shigella* with one percent hNGP did not affect its viability under the conditions tested. As expected, increasing concentrations of this extract resulted in decreasing bacterial viability. At 10% treatment, 40-50% of the inoculum survived (Figure 6A). We incubated *Shigella* with sub-lethal concentrations of hNGP before infection and then determined that adhesion to HeLa cells increased from 1 to 14 % in a dose dependent manner (Figure 6B). *Shigella* adhesion precedes invasion, which we tested in a gentamycin protection assay. Incubation of *Shigella* with hNGP increased invasion up to 30 fold in a dose dependent manner (Figure 6C). Similar results were obtained with the human intestinal epithelial cell line HT-29, suggesting that hyperinvasion does not depend on a specific epithelial host cell (data not shown).

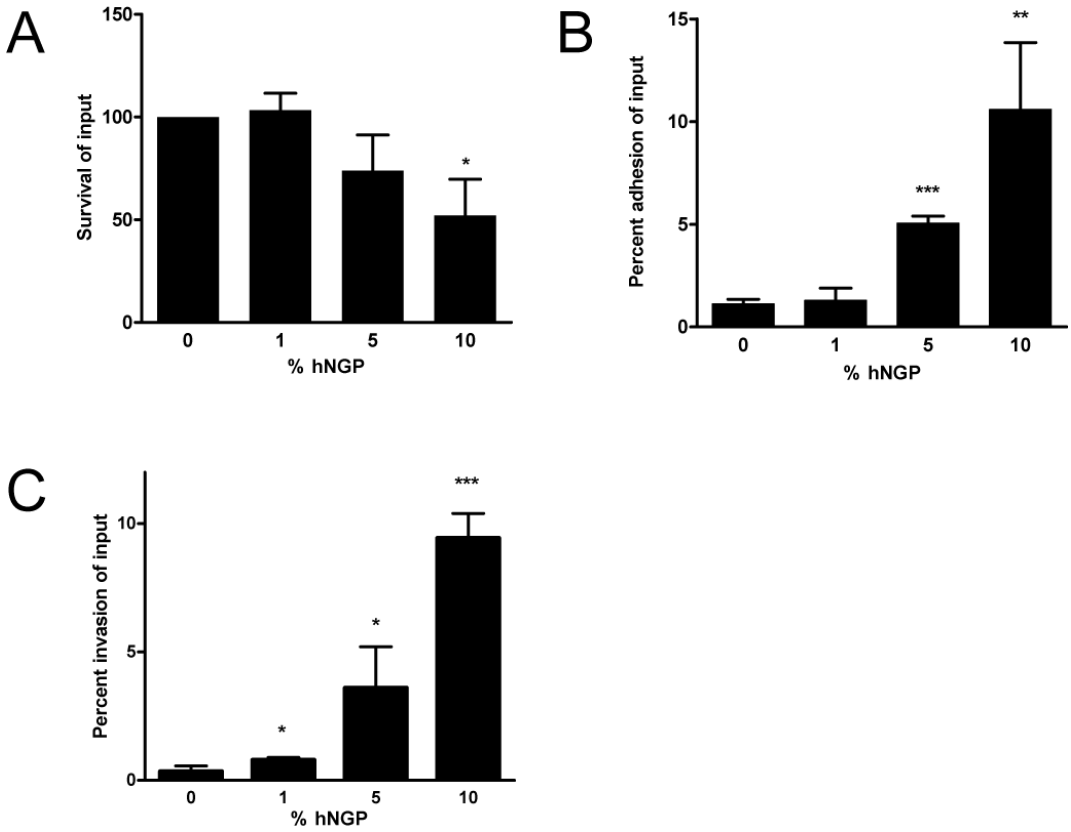

Figure 6: hNGP pre-treatment enhances *Shigella* adhesion and invasion at sub-lethal concentrations.

(A) Effect of hNGP pre-treatment on *Shigella* viability. *Shigellae* were treated with the indicated concentrations of hNGP for 15 minutes at 37°C. After washing we plated and determined the Colony Forming Units (CFU)/ml. Results are represented as the percentage of input bacteria and are the means +/- SD of three independent experiments. hNGP treatment induces killing up to 60%. **(B)** *Shigellae* pre-treated with the indicated concentrations of hNGP (%vol/vol) were centrifuged onto HeLa cells (MOI 100:1) to synchronize the infection. After washing, the cells were lysed with 1% Triton/PBS and the CFU enumerated after plating. Adhesion was defined as the total number of HeLa cell- associated bacteria and is shown as the percentage of input. Adhesion increased for M90T when *Shigella* was pre-treated with hNGP. Results of three independent experiments are shown as mean ± SD.**(C)** HeLa cells were infected with *Shigella* pre-treated with the indicated concentrations of hNGP (%vol/vol) at a MOI of 100:1 in a gentamycin protection assay. In brief, pre-treated bacteria were centrifuged onto the cells. After 30 minutes, extracellular bacteria were killed by the addition of gentamycin for 1 hour and the intracellular CFU was determined 1 hour after addition of gentamycin by plating. Invasion is shown as the

number of intracellular bacteria as percentage of input. Invasion is strongly increased with M90T (C) when cells were infected with hNGP treated *Shigella*. Results of three independent experiments are shown as mean ± SD.

Shigella uses virulence factors delivered by the TTSS to invade epithelial cells. To check if virulence factors are still required when invasion is more efficient due to hNGP treatment, we infected HeLa cells with BS176, a *Shigella* strain that lacks the virulence plasmid. BS176 showed increased adhesion (Figure 7A) but not invasion (Figure 7B) after treatment with hNGP. The fact that BS176 didn't show invasion after hNGP treatment shows that virulence factors are still required for invasion during hyperinvasion. Taken together, these data indicate that hNGP increases *Shigella* adhesion to the host cell, which in turn results in hyperinvasion.

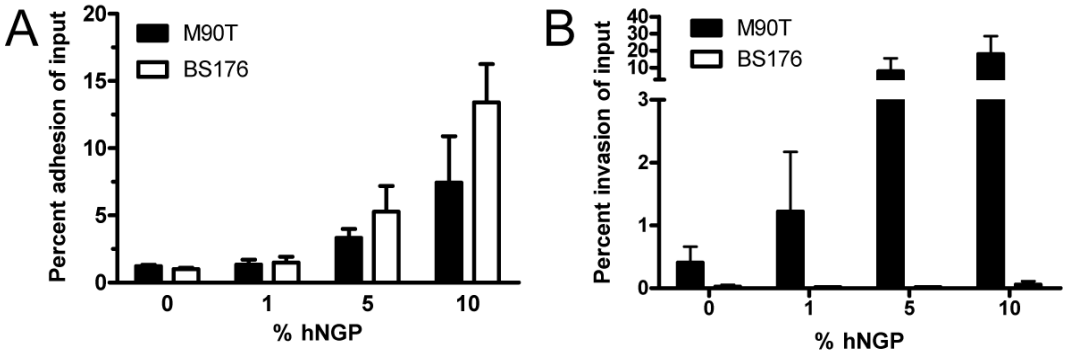

Figure 7: hNGP mediated increase of adhesion is independent of secreted virulence factors and the TTSS.

Wild-type *Shigella* (M90T) and *Shigella* lacking the virulence plasmid (BS176) were treated with hNGP and used to infect HeLa cells. Increased adhesion was observed with both M90T and BS176. Invasion during hyperinvasion depended on the presence of the virulence plasmid since BS176 did not show invasion upon hNGP treatment.

hNGP induced hyperinvasion independently of the multiplicity of infection (MOI) (Figure 8), indicating that hyperinvasion is not dependent on the number of infecting bacteria.

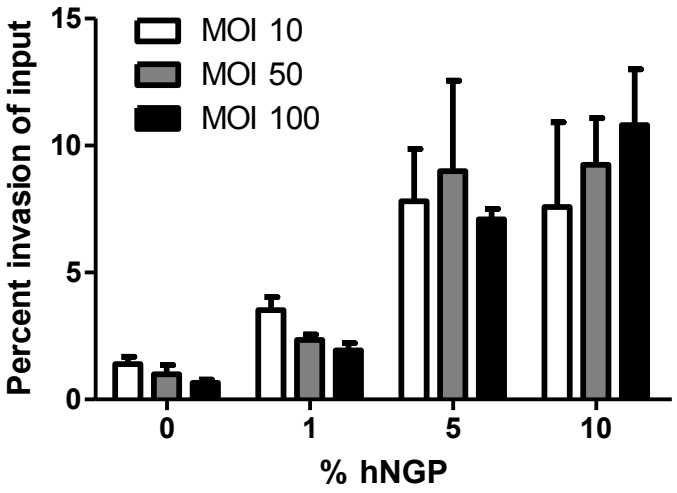

Figure 8: Hyperinvasion is independent of the multiplicity of infection.

HeLa cells were infected with hNGP treated *Shigella* at different MOI. One hour after infection, intracellular bacteria were enumerated as described. Hyperinvasion occurs at different MOIs. Data are representative of two independent experiments and show the mean of triplicates +/- SD.

3.1.2 Neutrophil degranulation causes hyperinvasion

Next, we analyzed whether degranulation of isolated neutrophils promotes hyperinvasion. We stimulated neutrophils seeded in the upper chamber of a transwell system to degranulate with the bacterial peptide fMLF (N-formyl-methionyl-leucyl-phenylalanine). The pore size of the transwell system used allowed diffusion of degranulated proteins but not transmigration of neutrophils. One hour after stimulation, we added *Shigellae* to the lower chamber for 30 minutes to allow them to mix with the degranulated proteins and then determined invasion in a gentamycin protection assay on HeLa cells already present in the lower chamber (Figure 9). Bacteria viability was dependent on the number of stimulated neutrophils and decreased by up to 45 to 55% (data not shown). Notably, upon neutrophil stimulation, *Shigella* invasion efficiency increased up to 30-fold and this

depended on the number of neutrophils in the upper chamber. As expected, unstimulated neutrophils did not induce hyperinvasion. These data show that proteins released by neutrophils through degranulation are sufficient to induce hyperinvasion.

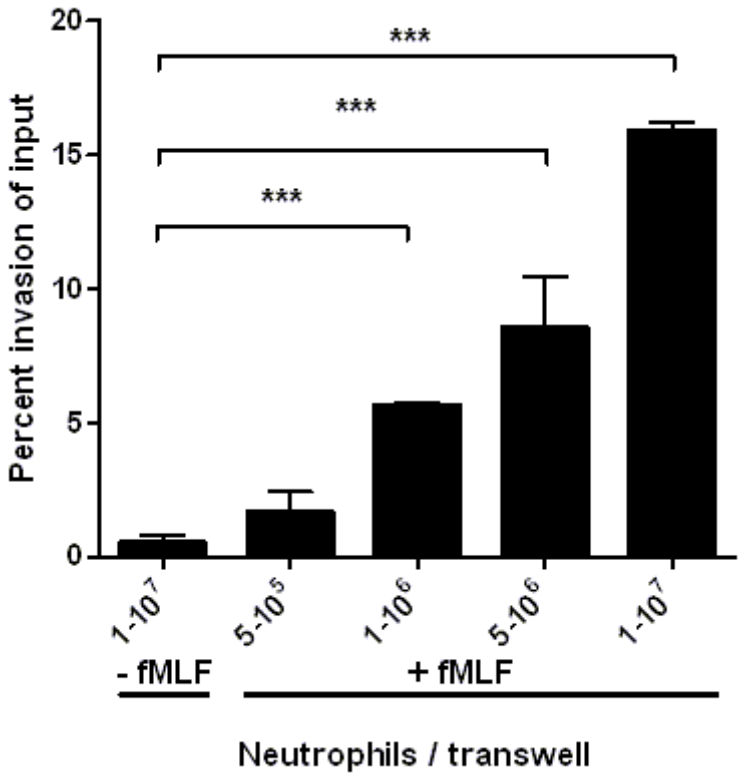

Figure 9: Degranulation of isolated neutrophils causes hyperinvasion.

Increasing numbers of isolated human neutrophils were stimulated with 1 µM fMLP for 1 hour to degranulate in the upper chamber of a transwell system. After degranulation, neutrophils were removed and bacteria were added to the lower chamber. Following incubation with the degranulated proteins intracellular bacteria were enumerated in a gentamycin protection assay. The unstimulated control contained $1 \cdot 10^7$ neutrophils. This shows that degranulation of neutrophils causes hyperinvasion. Results are shown as a percentage of input bacteria and a representative of three experiments is shown as mean ± SD

3.1.3 hNGP protein binding to *Shigella* causes hyperinvasion

Pre-incubating HeLa cells with up to 10% hNGP did not result in hyperinvasion (Figure 10A), suggesting that the granular proteins associate with the *Shigella* surface and not the host cell. Since hNGP enhances adhesion of wild type and avirulent *Shigella*, we proposed the hypothesis that the increase of adhesion is independent of *Shigella* virulence factor secretion and is caused by changes in the charge of the *Shigella* surface after binding of granular proteins. If this was the case, removing bound granular proteins should inhibit hyperinvasion. To test this, we first pretreated *Shigella* with hNGP and then eluted the bound proteins with 200 mM $MgCl_2$, competing them out of the bacterial surface (Weiss, et al., 1983). Incubation with $MgCl_2$ did not affect invasion efficiency of untreated *Shigella*. However, protein elution from the surface reduced hyperinvasion (Figure 10B), indicating that granular proteins have to be present on the surface of *Shigella* and not the host cell to cause hyperinvasion.

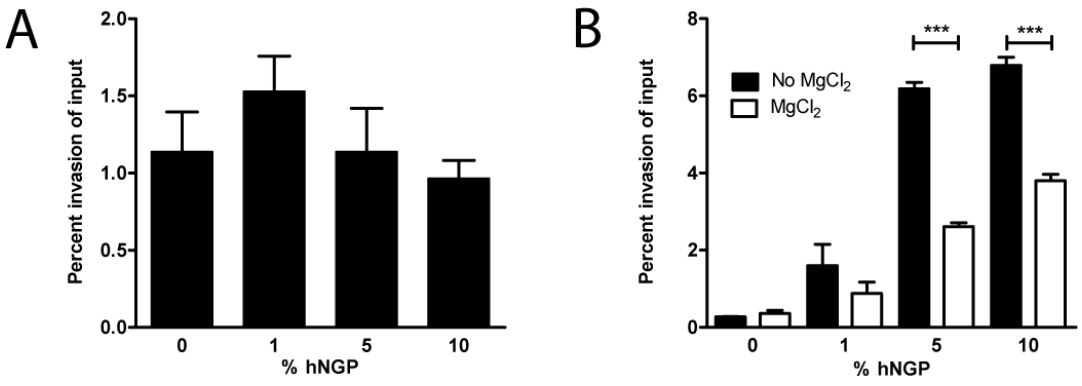

Figure 10: hNGP binding to bacteria causes hyperinvasion.

(A) hNGP treatment of host cells does not cause hyperinvasion. HeLa cells were pre-treated with hNGP at the indicated concentrations for 15 minutes, washed once with DMEM and then infected. Intracellular bacteria were quantified with a gentamycin protection assay. Results are representative of two independent experiments. Data are shown as mean of triplicates ± SD. (B) Elution of hNGP proteins from the surface of the bacteria reduces *Shigella* hyperinvasion. *Shigella* were pre-treated with hNGP at the indicated concentrations and then incubated either in PBS or PBS with 200 mM $MgCl_2$ to elute hNGP proteins. After the treatment, bacterial invasion was measured in a gentamycin protection assay. Results are representative of three independent experiments. Data shown are mean of triplicates ± SD.

3.1.4 Bactericidal Permeability Increasing protein (BPI), hCap18 and LL37 bind to *Shigella* after hNGP exposure but only BPI induces hyperinvasion

To identify the hNGP protein(s) responsible for hyperinvasion, we incubated *Shigella* pre-treated with hNGP with high concentrations of $MgCl_2$ as described above. Eluted proteins were concentrated, resolved by 1D-gel

electrophoresis and silver stained (Figure 11A).

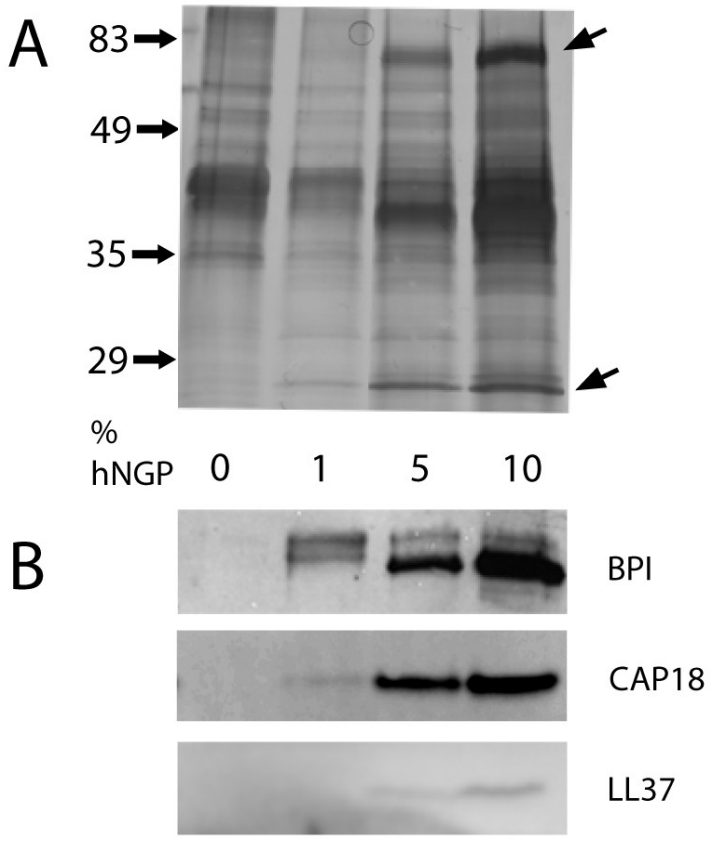

Figure 11: hNGP proteins bind to *Shigella* during hNGP treatment

Bacteria (10^8) were incubated in the absence (0%) or presence of increasing concentrations of hNGP at the indicated concentrations in PBS for 15 minutes at 37°C. At the end of the incubation period, the bacteria were centrifuged, washed once with PBS to remove un-adsorbed hNGP and the bound proteins were eluted from the cell pellet with 200mM $MgCl_2$. Eluates were collected by centrifugation, precipitated by TCA, subjected to SDS-PAGE and silver stained. Molecular mass standards (in kDa) are indicated. An increase in putative bacteria-associated protein species (arrows) is observed after increasing hNGP treatment. (B) Binding of BPI, hCap18 and LL-37 to *Shigella* was demonstrated by western blot analysis of eluted proteins. A representative of three experiments is shown.

Binding of specific proteins (Arrows in figure 11A) to *Shigella* was dependent on the hNGP concentration. The molecular weights of the most ab-

undant proteins suggested that they were BPI and hCAP18 (50-60 kDa and 18 kDa). Western blot analysis showed that BPI and hCAP18 bind to *Shigella* (Figure 11B). Both of these proteins are abundant in neutrophil granules and are released by degranulation (Weiss and Olsson, 1987); (Marra, et al., 1992) In addition, we confirmed the binding of LL-37, a 37 amino acid long cleavage product of hCAP18 (Sorensen, et al., 2001), to *Shigella*. Pre-treating *Shigella* with 1-5 µg / ml of recombinant BPI, all sub-lethal concentrations, increased invasion up to 25 fold compared to an un-treated control (Figure 12A). In contrast, incubation with LL-37 alone did not increase invasion (data not shown). Interestingly, BPI and LL-37 acted synergistically since invasion increased 130 fold when *Shigella* was pre-incubated with 10 µg / ml BPI and 5 µg / ml LL-37 (Figure 12A). To test if any LPS binding protein induces hyperinvasion, we incubated *Shigella* with LPS Binding Protein (LBP), a serum protein with sequence and structural similarity to BPI (Beamer, et al., 1997). Notably, LBP did not cause hyperinvasion (Figure 12B) at concentrations between 0.5 and 5 µg / ml suggesting that BPI has effects other than just binding LPS. In contrast to the cationic BPI and LL-37, LBP is neutral. This suggested that positive charges are required in proteins to induce hyperinvasion. Therefore we treated *Shigella* with poly-lysine (Figure 12C) or poly-arginine (data not shown), both artificial cationic polypeptides, and determined invasion. Both polypeptides induced hyperinvasion at concentration between 1 and 5 µg / ml, suggesting that the effect of the neutrophil cationic proteins is mediated by charge.

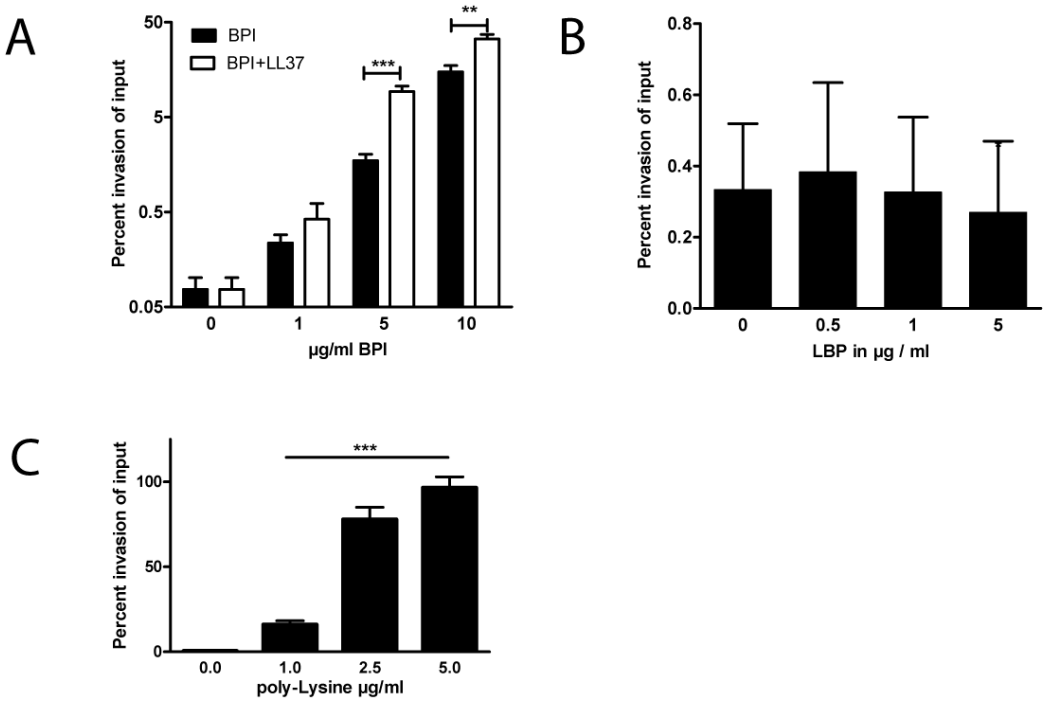

Figure 12: Specific neutrophil proteins induce hyperinvasion

(A) *Shigellae* were pre-treated with different concentrations of BPI and / or LL-37 as described in Materials and Methods and used in a gentamycin protection assay. BPI induces hyperinvasion and LL-37 has a synergistic effect. Note that the scale in the y-axis is logarithmic. Results are representative of two independent experiments. Data represent mean of triplicates ± SD. (B) Treatment of *Shigella* with increasing concentrations of LPS binding protein (LBP), a close homologue of BPI, did not induce hyperinvasion. *Shigellae* were pre-treated with the indicated concentrations of LBP in PBS for 15 minutes at 37°C with slow shaking and invasion determined in a Gentamycin protection assay. Results are represented as a percentage of input bacteria and are the means +/- SD of triplicate samples of three independent experiments. (C) The artificial poly-peptide poly-lysine induces hyperinvasion. *Shigellae* were pre-treated with the indicated concentrations of poly-lysine in PBS for 15 minutes at 37°C with slow shaking and invasion determined in a Gentamycin protection assay. These data show that cationic proteins induce hyperinvasion. Results are representative of two independent experiments. Data represent mean of triplicates ± SD.LPS mutants with a negative surface charge show increased hyperinvasion

3.1.5 LBP and LL-37 do not influence adhesion of Shigella

LL-37 did not induce hyperinvasion (data not shown) as well as LBP (Figure 12). To exclude that these proteins influence adhesion, we infected HeLa cells with *Shigella* pre-treated with increasing concentrations of either LBP or LL-37 and measured adhesion. We did not detect any change in adhesion (Figure 13), indicating that LL-37and LBP do not prevent adhesion and thereby hyperinvasion.

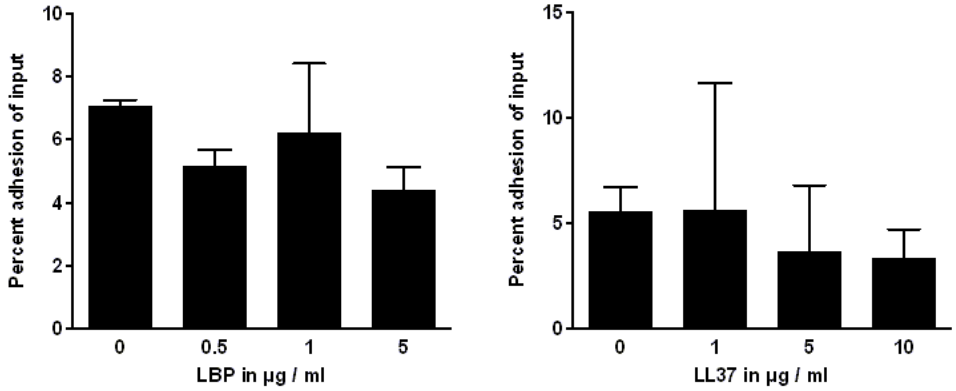

Figure 13: Pre-treatment of *Shigella* with LBP or LL-37 does not change adhesion.

Shigella were pre-treated with increasing concentrations of LBP or LL-37 and adhesion to HeLa cells was analyzed as described. There was no change in the adhesion of *Shigella* to HeLa cells.

3.1.6 LPS mutants with a negative surface charge show increased hyperinvasion

We reasoned that binding of hNGP to the surface of *Shigella* might enhance adhesion by facilitating electrostatic interaction between cationic proteins of the hNGP and the negative surface of the host cell. Therefore, we tested whether *Shigella* LPS mutants with different surface charges are hyperinvasive after hNGP treatment. We analyzed mutants in the genes

gtrA, *gtrB*, *rfbA*, *waaL*, and *waaJ*, all of which are defective in rabbit ileal loop colonization (West, et al., 2005). Mutants *ΔwaaL* and *ΔwaaJ* have a negative surface charge while mutants in *ΔgtrA*, *ΔgtrB*, *ΔrfbA* are neutral (Geldmacher and Chaput, unpublished observations). *ΔgtrA, ΔgtrB, ΔrfbA* showed normal invasion and hyperinvasion (data not shown). Untreated ΔwaaL was slightly less invasive than wild-type *Shigella*, but adhesion was not affected. *ΔwaaL* (Figure 14A) and *ΔwaaJ* (data not shown) were more hyperinvasive than wild-type *Shigella*. *ΔwaaL* adhered about 5-10 fold more efficiently (Figure 14A) and was 2-3 fold more invasive (Figure 14B) than wild-type *Shigella* M90T when exposed to hNGP. These results show that *Shigellae* carrying a negative surface charge are more hyperinvasive when treated with hNGP than wild-type *Shigella*, indicating that changes in the surface charge induced by hNGP lead to increased adhesion to and invasion of *Shigella* to host cells.

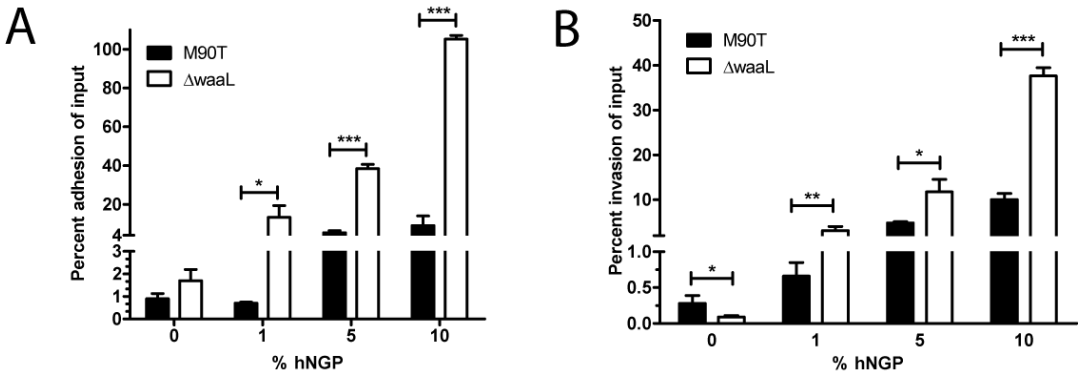

Figure 14: Surface charge determines hyperinvasion

Wild-type and *ΔwaaL*, a mutant in the gene that encodes the o-antigen ligase, were treated with hNGP before testing adhesion (A) and invasion (B). Both of these phenotypes are more pronounced in *ΔwaaL*, indicating that charge is essential for the effects of hNGP. Results are representative of two independent experiments. Data shown are means of triplicates ± SD.

3.1.7 hNGP treatment does not change hydrophobicity of *Shigella*

In addition to surface charge, hydrophobicity influences adherence of bacteria to their host cell. Therefore, we checked the hydrophobicity of hNGP treated *Shigella* using two different approaches. First, we used hydrophobic interaction chromatography (HIC) which measures binding to hydrophobic sepharose at different salt concentrations. Since both of these methods require a large number of bacteria, we treated *Shigella* with 5% hNGP to reduce the amount of hNGP needed. We treated *Shigella* in the same way as we did for adhesion and invasion assays. Treatment with 5% hNGP did not lead to any detectable changes in hydrophobicity (Figure 15A). We confirmed this data using an alternative approach. Here, Microbial Adhesion To Hydrocarbon (MATH) measures the transition of bacteria from the water phase to the organic phase after vortexing. The more hydrophobic bacteria are, the more efficient they enter the organic phase. Again, we were not aible to detect any changes in hydrophobicity confirming our results (Figure 15B).

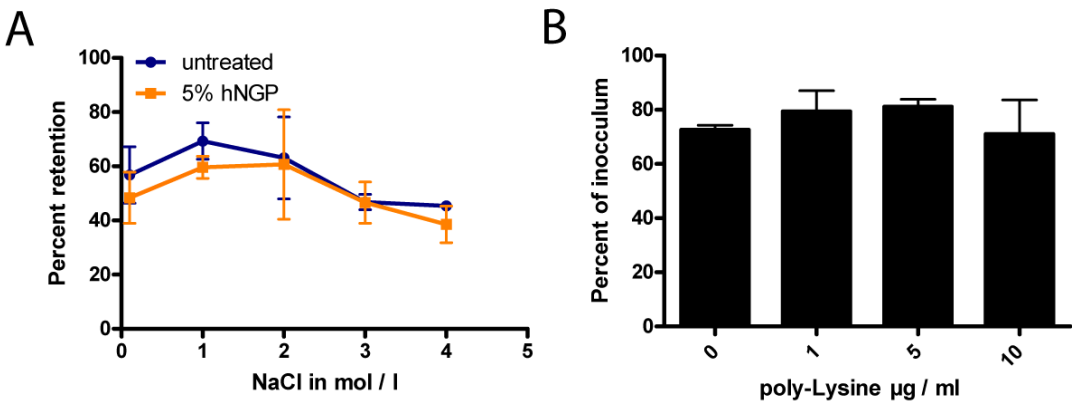

Figure 15: Hydrophobicity of Shigella does not change upon hNGP treatment.

Shigella were pre-treated with hNGP (A) or poly-lysine (B) and tested for changes in hydrophobicity either by (A) Hydrophobicity interaction chromatography (HIC) or (B) Microbial Adhesion To Hydrocarbon (MATH). There were no significant changes in hydrophobicity after hNGP treatment. This indicates that changes in hydrophobicity don't contribute to increased adhesion of *Shigella* to its host cell.

3.2 IL-8 inhibition

3.2.1 hNGP treatment inhibits IL-8 secretion

Shigella invasion of HeLa cells causes IL-8 secretion. Since hyperinvasion increases the number of intracellular bacteria, we tested IL-8 secretion by infected cells. IL-8 secretion was measured by ELISA at three hours post infection. At this timepoint, IL-8 secretion is maximal and *Shigellae* are still growing inside the cells even when treated with hNGP (Figure 16).

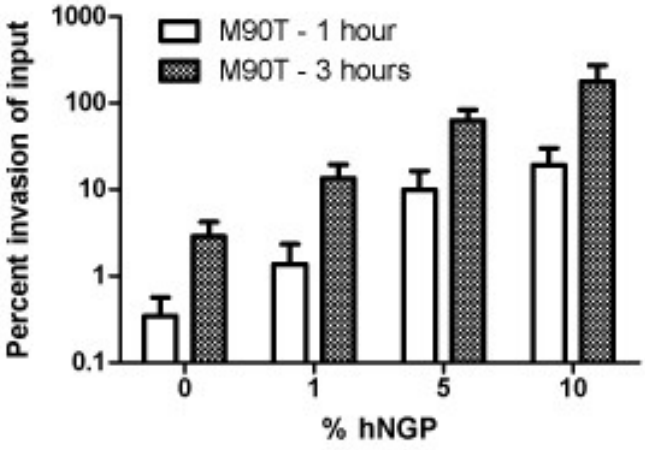

Figure 16: Hyperinvasive *Shigella* grow intracellularly after invasion

HeLa cells were infected with hNGP treated *Shigella* as described before. The number of intracellular bacteria was determined using a gentamycin protection assay. Cells were lyzed and bacteria were plated at 1 and 3 hours gentamycin treatment. Note the log scale of the graph.

Surprisingly, IL-8 concentrations in the supernatant did not increase with the number of intracellular bacteria, but rather decreased down to about one third of IL-8 secreted by cells infected with untreated *Shigella* (Figure 17A).

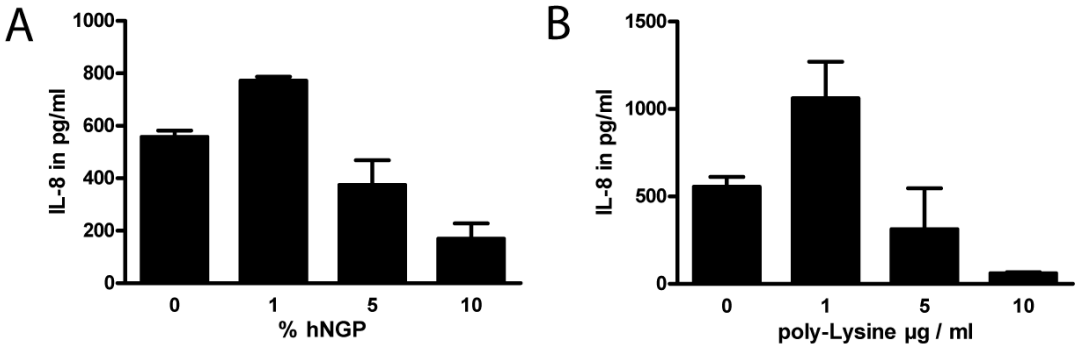

Figure 17: hNGP pre-treatment and poly-lysine pre-treatment inhibit IL-8 secretion by infected HeLa cells.

HeLa cells were infected with hNGP pre-treated *Shigella* as described before. After three hours of infection, supernatants of infected cells were removed and analyzed for IL-8 by ELISA. Although increasing numbers of intracellular bacteria are present, the amount of secreted IL-8 decreases with increasing concentrations of hNGP or poly-lysine.

This was also true for hyperinvasion induced by pre-treating *Shigella* with poly-lysine (Figure 17B) before infection. Here, inhibition was more pronounced, correlating with the stronger induction of hyperinvasion of poly-lysine compared to hNGP (Figure 12C).

3.2.2 IL-8 inhibition is achieved by inhibition of NF-kB and MAPK signaling

IL-8 expression depends on the activity of several signaling molecules. NF-kB activation is absolutely required for IL-8 expression. We examined NF-kB activity using a dual luciferase reporter system. In this assay, the cells are transfected with a plasmid carrying the firefly luciferase under the control of a NF-kB activated promoter. To normalize, renilla luciferase is expressed constitutively from a co-transfected plasmid. We observed a dose dependent inhibition of the NF-kB driven firefly luciferase expression (Fig. 18B, -TNF-α). In addition to NF-kB activation, MAP kinases contribute to IL-8 expression levels. Therefore we tested the phosphorylation of Erk1/2, p38 and Jnk in HeLa cells infected with hNGP and poly-lysine treated *Shigella* using antibodies specific for the phosphorylated forms of these kinases. We observed inhibition of phosphorylation of p38 and Erk1/2 but not of Jnk (Figure 18A, -TNFα). To test whether this inhibition is reversible, we activated the NF-kB pathway by incubating the cells with TNF-α. TNF-α treatment before infection caused an overall increase of IL-8 secretion in all infected cells. However, we still observed inhibition of phosphorylation of MAP Kinases by hNGP pre-treated *Shigella* (Fig 18A, +TNF-α). This inhibition was also present on the level of NF-kB. All of these results were also reflected by secreted IL-8 levels which were de-

tected by ELISA (Figure 18C). Taken together, these data show that activation of NF-kB is inhibited by infection with hNGP treated *Shigella*. In addition, TNF-α pre-stimulation activates NF-kB but is also inhibited with increasing hNGP concentrations. Poly-lysine pre-treatment is a strong inhibitor of all pathways which are affected by hNGP pre-treatment.

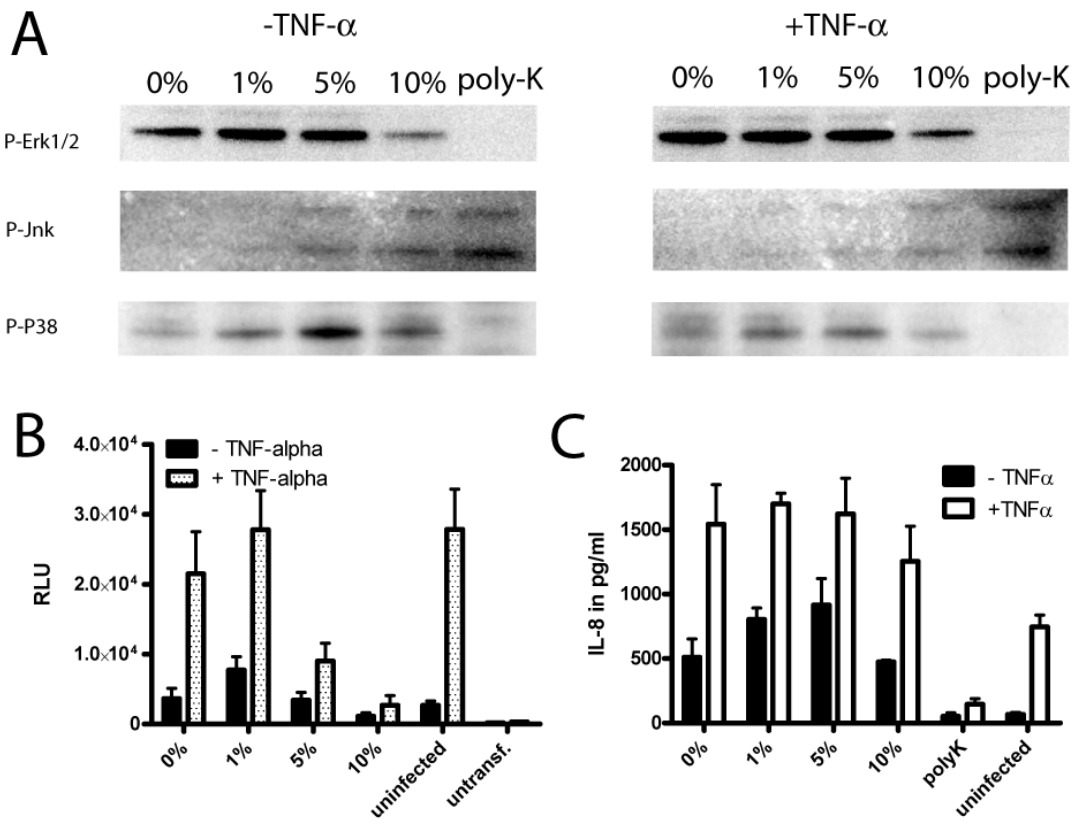

Figure 18: Inhibition of signalling pathways required for IL-8 expression.

(A) HeLa cells were infected with *Shigella* pre-treated with either hNGP or poly-lysine. Three hours after infection, cells were washed and analyzed by western blot for phosphorylated forms of Erk1/2, p38 and Jnk. Jnk, shows increasing phosphorylation with increasing concentrations of hNGP or with 5 µg/ml poly-lysine. (B) HeLa cells were transfected with a NF-kB driven firefly luciferase and infected with hNGP or poly-lysine treated *Shigella*. Activation of NF-kB was measured in a luminometer. Firefly levels are shown as relative light units (RLU). (C) Levels of secreted IL-8 were detected by ELISA.

3.2.3 Hyperinvasion causes accelerated cell death

We reasoned two possible mechanisms for hNGP mediated inhibition of IL-8 secretion. First, inhibition could be caused by accelerated cell death of the infected host cell. Second, hNGP present on the surface of the bacteria might prevent recognition of the respective peptidoglycan ligands by Nod1 and Nod2, resulting in no activation of the NF-kB and MAPK path-

ways. *Shigella* has been shown to induce necrotic cell death in non-myeloid cells including mitochondrial damage which can be counterbalanced by Nod1 dependent NF-kB activation (Brinkmann, et al., 2004; Carneiro, et al., 2009). Therefore, we tested at what time cell death occurs after infection of HeLa cells with hNGP or poly-lysine pre-treated *Shigella*. Cell death was monitored by two different assays. We used Sytox green and lactate dehydrogenase (LDH) release assays to measure plasma membrane integrity. While Sytox green is a small dye that incorporates into DNA upon membrane leakage, LDH is a 25 – 40 kDa (depending on the isoform used) protein which can only be released from cells with profound membrane instability. We monitored cell death over several hours after infection in a CO_2 incubator. Interestingly, cells became sytox positive only when infected with hNGP pre-treated *Shigella* (Figure 19A). HeLa cells infected with untreated *Shigella* remained viable until 10 hours after infection. Induction of cell death showed dose dependency with regards to hNGP treatment (Fig 19A). The same was true for poly-lysine (data not shown). The first signs of cell death could be observed at 3 hours in cells infected with *Shigella* pretreated at 10% hNGP. Lower concentrations showed cell death with delayed kinetics. Cell death induced by *Shigella* treated with 1% hNGP was not significant in this assay during the observed time period. Interestingly, LDH release showed a very similar pattern but with a delay of about one hour (Figure 19B). Here, we followed cell death until 5 hours after infection. LDH release was more profound by infection with poly-lysine treated *Shigella*.

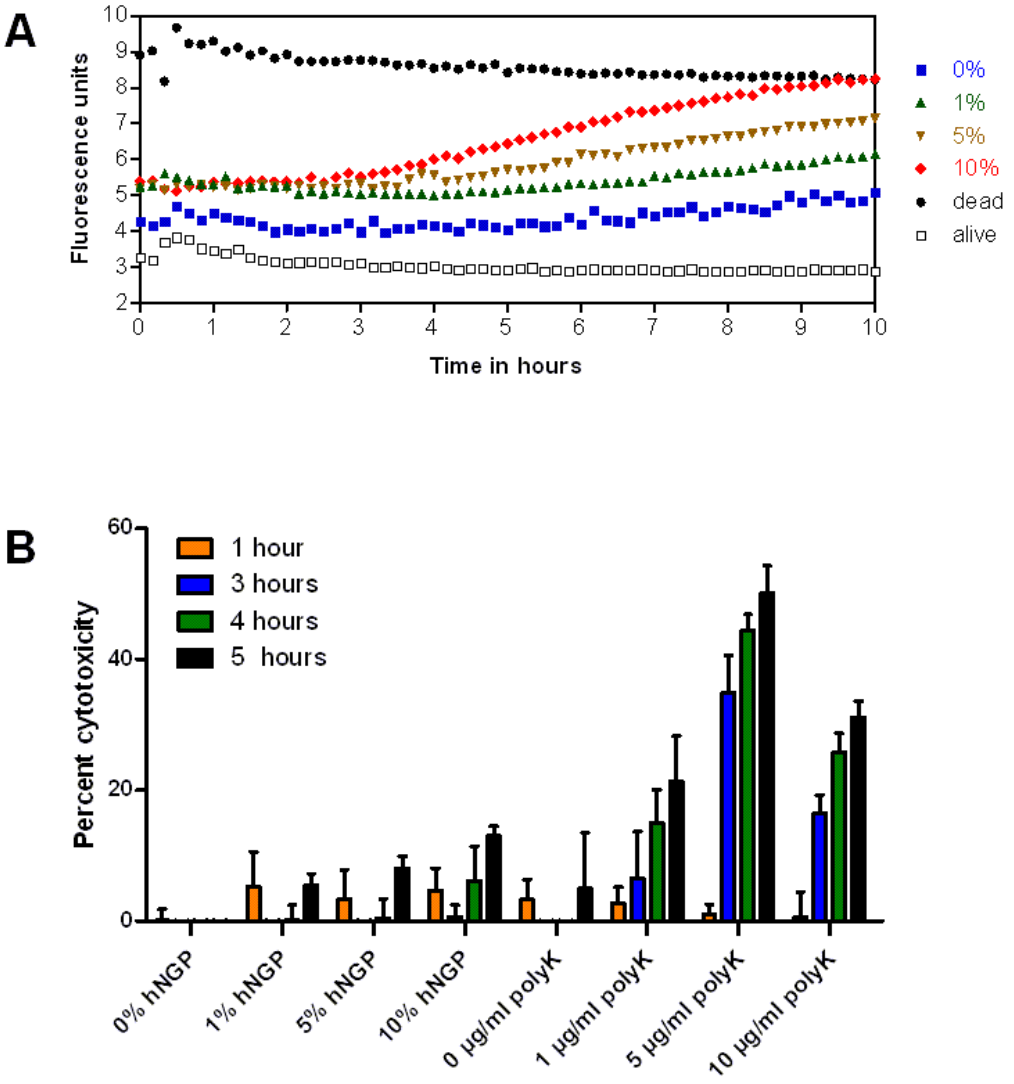

Figure 19: Infection with hNGP or poly-lysin pre-treated Shigella causes cell death.

HeLa cells were infected with hNGP or polyK pre-treated *Shigella* in the presence of sytox in a CO_2 incubator at 37°C. Supernatant samples for LDH release assay were taken at the indicated time points. (A) Cells became sytox positive in a dose dependent manner from 3 hours on. (B) LDH release was significant about 1 hour after cells became sytox positive. For example at 5% hNGP, the sytox signal raises about 4 hours after infection. With regards to LDH release, at 5% significant LDH release can be measured at 5 hours after infection.

3.2.4 Apoptosis is not involved in accelerated cell death caused by hyperinvasion

HeLa cells infected with hNGP treated *Shigella* were also checked for induction of apoptosis. We stained infected cells for nicks in their nuclear DNA using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) technique. There were no signs of apoptosis in infected cells at 1 hour (data not shown) and at 3 hours (Figure 20), indicating that hyperinvasion did not lead to apoptosis at all hNGP concentrations up to three hours post infection.

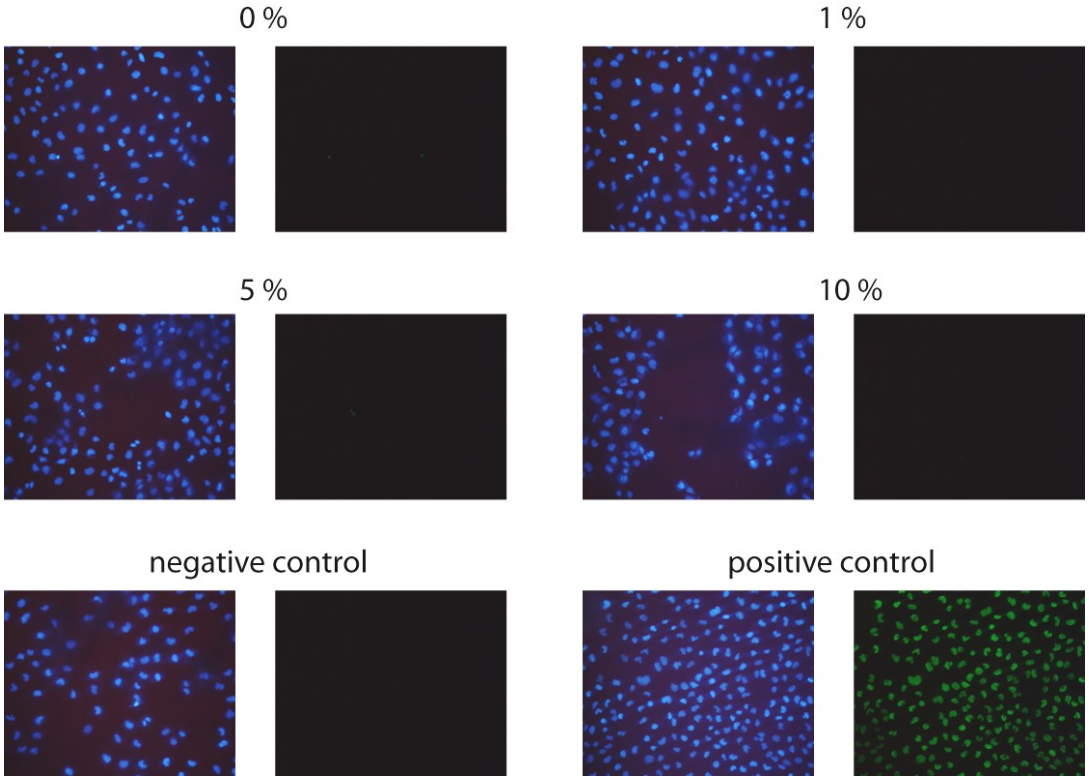

Figure 20: Infection with hNGP treated Shigella does not trigger apoptosis up to three hours

HeLa cells were infected with hNGP treated *Shigella* at indicated concentrations. Apoptosis was detected by TUNEL labelling. There was no signifi-

cant increase in TUNEL$^+$ compared to the negative control cells at one (data not shown) and three hours after infection.

3.2.5 IL-8 inhibition is not mediated by OspF, OspG, BipA and IpaH 9.8

Several effectors secreted by *Shigella* are possible candidates for down-regulation of IL-8 secretion. These were selected based on their expression profile for example in response to invasion or contact of antimicrobial proteins with the bacterial surface. OspF has been reported to be a phosphothreonine lyase (Li, et al., 2007) or a dephosphorylating enzyme causing inhibition of the extracellular regulate kinase (Erk) in the nucleus leading to modified histone phosphorylation which results in reduced NF-kB activation (Arbibe, et al., 2007). Recent publications describe the exact mechanism of the Salmonella homologue of OspF, SpvC to be of the phospholyase type (Zhu, et al., 2007).

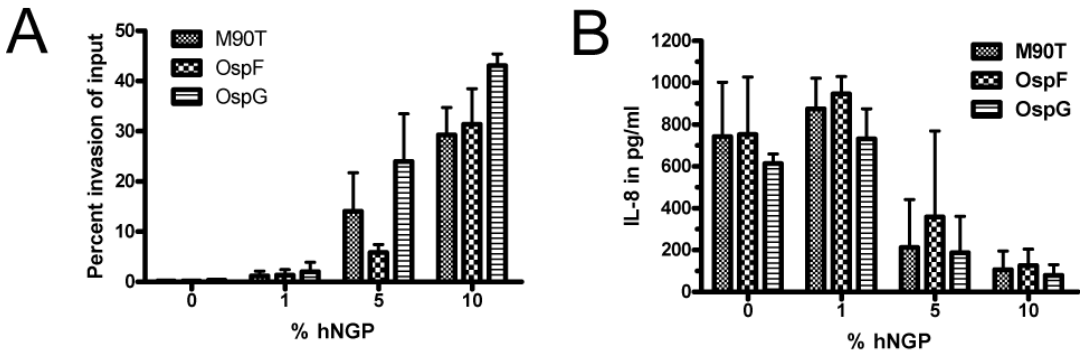

Figure 21: Antiinflammmatory second wave effectors OspF and OspG are not involved in IL-8 downregulation.

HeLa cells were infected with hNGP pre-treated *Shigella* and invasion (A) at 1 hour as well as (B) IL-8 secretion at three hours after infection was measured. There was no significant difference in invasion (except for 5% hNGP OspF) or IL-8 secretion. This shows that OspF and OspG are not responsible for downregulation of IL-8.

OspG antagonizes degradation of the inhibitor IκBα by blocking its ubiquitinylation (Kim, et al., 2005) which also leads to reduced activation of NF-kB. Mutants of these effectors did not show any difference with respect to IL-8 inhibition (Figure 21B) while the number of intracellular bacteria was not affected (Figure 21A). BipA stands for BPI induced protein A. Expression of this effector is upregulated after contact of *Salmonella* with BPI. Since BPI induces hyperinvasion, this made BipA an excellent candidate protein. Therefore, we knocked out BipA using the Datsenko-Wanner method (Datsenko and Wanner, 2000) and tested for its ability to be hyperinvasive upon hNGP treatment as well as downregulation of IL-8 secretion. As shown in figure 22, *Shigella*e lacking BipA were still able to inhibit IL-8 secretion in infected HeLa cells. IpaH9.8 is one of the effectors regulated by IpgC and is secreted after the Ipa proteins. It acts as an E3 ligase binding the splicing factor U2AF(35) which causes reduction in the transcription of proinflammatory genes such as Il-8, RANTES, GM-CSF, and Il-1beta (Okuda, et al., 2005; Rohde, et al., 2007).

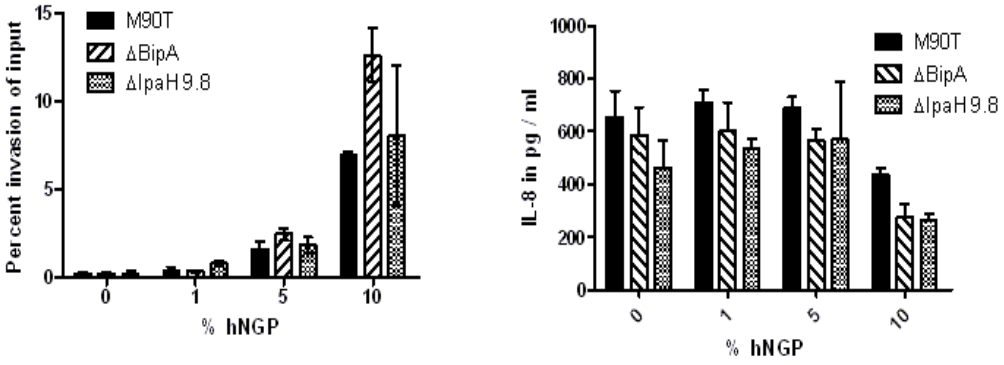

Figure 22: ΔBipA and ΔIpaH9.8 inhibit IL-8 secretion.

HeLa cells were infected with hNGP pre-treated *Shigella* and invasion (left panel) at 1 hour as well as IL-8 secretion (right panel) at three hours after infection was measured. There was no significant difference in invasion or IL-8 secretion. This shows that BipA and IpaH9.8 are not responsible for downregulation of IL-8.

We also knocked out IpaH9.8 and tested for IL-8 secretion and found no effect on IL-8 inhibition (Figure 22). In addition, we tested the mutants ΔshiA and ΔIpaH7.8 available in the lab which were known to have anti-inflammatory functions and these didn't show a difference in IL-8 inhibition as well (data not shown). Most of these proteins are regulated by MxiE. Therefore we also tested an *mxiE* mutant to check for all the involvement of all proteins regulated by MxiE, but this mutant nicely inhibited IL-8 in cells infected with hNGP treated *Shigella* (data not shown). This showed that none of our candidates analyzed inhibits IL-8 secretion in our test system.

4 Discussion

4.1 Hyperinvasion

Neutrophils are the first cells to arrive at the site of infection during Shigellosis. Resident macrophages residing in lymphoid follicles underlining the epithelium cannot contain *Shigella* inside their phagosome and die when cytoplasmic *Shigella* activates caspase-1. In contrast, neutrophils prevent the escape of *Shigella* from the phagosome, because neutrophil elastase specifically cleaves virulence factors from pathogenic bacteria (Weinrauch, et al., 2002). Besides phagocytosis, NET formation is able to trap and kill *Shigella* (Brinkmann, et al., 2004). Therefore, neutrophils are the first serious threat to *Shigella* by the host immune system during Shigellosis. In early stages of infection, neutrophils are attracted to the side of infection by a gradient of chemokines, anaphylatoxins and bacterial products. To reach the site of infection, they transmigrate through the vascular endothelium in a CD18 dependent manner (Perdomo, et al., 1994). At this stage degranulation occurs when phagocytosis is not possible due to the lack of physical contact to the bacteria. During this phase, *Shigella* is exposed to granular proteins released into the extracellular space. Simulating this exposure by treatment of *Shigella* with hNGP, we observe killing of 40-50 percent of the bacteria. Although this killing is significant, the remaining viable bacteria are highly invasive because of increased adhesion to the host cell. Exposure to neutrophil granular antimicrobial proteins enables *Shigella* to adhere non-phagocytic epithelial cells with much higher efficiency (Figure 6). *Shigella* LPS has been shown to act as an adhesin during invasion of guinea pig epithelium (Izhar, et al., 1982). The hyaluronic acid receptor has also been reported to be required for *Shigella* invasion (Skoudy, et al., 2000). However, these data do not contradict each other. LPS could be important for establishing contact to the host cell and then more specific binding mediated by receptor-ligand interaction takes place.

During adhesion, electrostatic interactions mediate contact over mid range distances of few 10-20 nm (Figure 23). Very tight and close adhesion is usually mediated by receptor-ligand interactions. Therefore, both LPS – cell surface and CD44-IpaB interactions might be important for the adhesion of *Shigella* to its host cell.

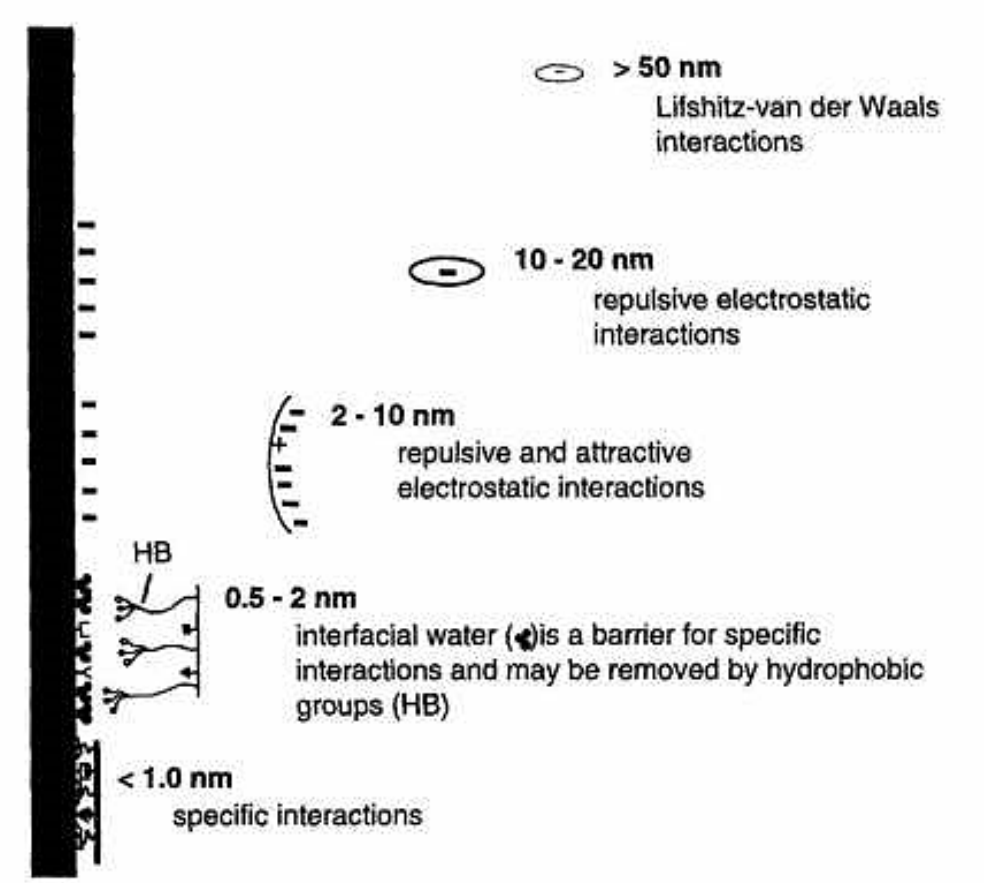

Figure 23: Physical interactions during adhesion

Adhesion is influenced by different forces depending on the distance between bacteria and the surface (taken from (Fletcher, 1996)).

4.1.1 In vivo relevance

One important question remaining is if hyperinvasion occurs *in vivo*. *Shigella* host specificity is very narrow. It infects only humans and primates via the oral route, while primates have to be infected with relatively high numbers in order to establish Shigellosis in contrast to humans (Takeuchi, et al., 1968). Orally infected mice don't show any symptoms of Shigellosis. However, *in vitro*, *Shigella* is able to infect a wide range of murine and human cell lines as well as primary cells. This indicates that the inability of *Shigella* to infect mice via the oral route is not because a specific receptor is lacking. It rather suggests existing but unknown physiological differences that prevent *Shigella* from reaching the epithelium of the murine intestine. There are more *in vivo* Shigellosis models, which are limited with respect to the infection site and the localization of the infection: The mouse lung infection model nicely resembles the proinflammatory response elicited by *Shigella* in a caspase-1 dependent manner (Sansonetti, et al., 2000). However, lung tissue shows a completely different organisation compared to the intestinal epithelium and subepithelial space (Wheater, et al., 1979). Therefore, the lung model is very well suitable to analyze the contribution of proinflammatory cytokines such as IL-1β and IL-18, but not of neutrophil degranulation to host cell invasion. Another model is the rabbit ileal loop model. Here, the rabbit ileum is ligated into small loops and microbes are injected into the lumen. This model resembles the initial stages of epithelial infection by *Shigella*. Using this model, several important observations concerning *Shigella* pathogenesis have been made, including its inability to infect epithelial cells from the apical side. Furthermore, M-cells have been described using this model as the route by which *Shigella* circumvents the epithelial barrier and reaches the basolateral side (Wassef, et al., 1989). After a few hours of infection, signs of inflammation such as swelling of the loop, massive neutrophil infiltration and erosion of

vili can be observed. All these symptoms can be prevented by injecting an α-CD18 antibody. Blocking CD18 prevents extravasation of neutrophils from the blood stream to the site of infection. Perdomo and collegues nicely showed that counts of *Shigella* in the epithelium and inflammation are reduced when neutrophils are prevented from extravasating (Perdomo, et al., 1994). Based on these data, they proposed that neutrophils opened the epithelial barrier by transmigration allowing a massive basolateral bacterial invasion. Our data suggest that besides this function, neutrophils recruited early in infection degranulate and release proteins that cause hyperinvasion. Hyperinvasion is caused by *Shigella* treated with a crude granular extract as well as granular proteins released form isolated neutrophils. We observed a similar induction of hyperinvasion with both sources of neutrophil granular proteins (Figure 9). This indicates that hNGP used for pre-treating *Shigella* is not biased towards certain proteins which induce hyperinvasion and could be isolated more efficiently during acid extraction. In addition, this shows that hyperinvasion is not an artifact of hNGP treatment but is also caused by live neutrophils after bacterial stimulation.

4.1.2 Proteins inducing hyperinvasion

We identified BPI from hNGP as a hyperinvasion inducing protein which is stored in azurophilic granules. During degranulation, azurophilic granules are mobilized last (Sengelov, et al., 1993). Our transwell experiments confirm that degranulation from isolated neutrophils provides sufficient amounts of BPI to induce hyperinvasion (Figure 9). Alternatively, there might be more unidentified granular proteins capable of causing hyperinvasion, since there are many more cationic AMPs present in the granules. We also showed that LL-37, a cationic granular AMP, does not induce hyperinvasion but shows synergy with BPI. LL-37 is present in specific granules that are released more easily during degranulation. Because of

their synergy in hyperinvasion, secretion of LL-37 might compensate for the lower amounts of BPI present because of limited azurophilic granule degranulation. Why BPI but not LL-37 induces hyperinvasion remains to be elucidated. LL-37 does neither prevent nor promote adhesion of *Shigella* to the host cell (Figure 13). BPI clearly induces hyperinvasion (Figure 12), but it remains to be analyzed if it also promotes adhesion. The crystal structure of both proteins is solved (Beamer, et al., 1997; Wang, 2008), showing a polar distribution of the positive charge for the BPI molecule and an overall positive charge of LL-37. Both proteins are highly cationic, the only difference being a more uniform distribution of the positive charge in LL-37. The fact that the artificial polypeptide poly-lysine strongly induces hyperinvasion underlines the importance of positively charged proteins for hyperinvasion. In addition, LBP - which has a neutral p.i. (9.4 for BPI and 6.3 for LBP, Gassteiger et al.). - didn't induce hyperinvasion and also did not enhance adhesion (Figure 12 and 13), although sharing about 50 % sequence identity with BPI in primary sequence. Another possible requirement for cationic proteins to induce hyperinvasion might be their molecular weight. BPI (50 kD) is considerably larger than LL37 (3.7 kD). The poly-lysine used has an average molecular mass of 75-150 kDa. LL-37 binds to *Shigella* (Figure 11), but might not cause hyperinvasion simply because it is not sufficiently exposed on the surface of *Shigella* to mediate adhesion to the host cell. This we are going to test by using poly-lysine of different molecular weight to treat *Shigella* and analyzing for hyperinvasion.

4.1.3 Mechanism of hyperinvasion

Although the characteristics of proteins inducing hyperinvasion are not completely clear, cationicity seems to be required. In addition, we observed that hyperinvasion was inhibited by removal of surface bound granular proteins (Figure 10) and host cell pretreatment did not cause hyper-

invasion (Figure 10. This indicated that binding of granular proteins changes the surface properties of *Shigella* enhancing their adhesion to the host cells. As already described, adhesion is influenced by electrostatic, hydrophobic and receptor-ligand interactions (Fletcher, 1996). In addition, LPS is important for *Shigella* host-cell interaction. Therefore, we tested mutants of *Shigella* with different LPS structures for their ability to be hyperinvasive. These mutants included deletions in the genes *gtrA, gtrB, rfbA, cld, waaD, waaJ* and *waaL,* all defective in colonization of rabbit ileal loops (West, et al., 2005). Mutants in *gtrA* and *gtrB* lack glycosylations in their O-antigen (Allison and Verma, 2000). The *cld* mutant lacks the mode B of the antigen, resulting in a shortened O-antigen of 15 repeats. The *rfbA* mutant cannot convert glucose-1-phosphate to deoxythymidine diphosphate–rhamnose (dTDP-rha), a step required for the efficient synthesis of O antigen. Deletion of the O-antigen ligase WaaL also leads to LPS without O-antigen. The genes waaD and waaJ encode proteins required for the synthesis of the LPS core (Kaniuk, et al., 2004). Of all these mutants, ΔwaaD, ΔwaaJ and ΔwaaL are negatively charged in contrast to wild-type *Shigella* which has a neutral surface charge (Geldmacher and Chaput, unpublished observations). ΔwaaD is non-invasive *in vitro* (data not shown) while ΔwaaJ (data not shown) and ΔwaaL show normal invasion but enhanced hyperinvasion (Figure 14). During adhesion, electrostatic interactions can either be repulsive or attractive. Electrostatic interactions between bacteria and their target cell are repulsive because most bacteria and cells are negatively charged (Carpentier and Cerf, 1993; Jucker, et al., 1996). Therefore, binding of cationic neutrophil antimicrobial proteins might change the surface charge thereby promoting initial interactions between the host cell and *Shigella*. Enhanced hyperinvasion observed with the ΔWaaL (Figure 14) and ΔWaaJ mutants might be caused by two effects. On one hand, the negative charge causes increased binding of cationic granular proteins which promote contact to the host cell.

Additionally, sterical hindrance by the O-antigen might be removed in these mutants, allowing easier access of granular proteins to Lipid A. If increased negative surface charge of *Shigella* indeed leads to increased binding of cationic proteins from neutrophil granules remains to be elucidated. With respect to hydrophobicity, we did not detect any changes by binding of hNGP to the surface (Figure 15). Either hNGP treatment does not change *Shigella* hydrophobicity, which would indicate that increased adhesion of *Shigella* solemnly depended on changed surface charge or the induced change was too subtle to be detected. In fact it has been reported that poly-lysine does indeed change hydrophobicity of bacteria (Goldberg, et al., 1990), however the concentrations used were much higher than in our experiments. Using these high concentrations, none of the bacteria would have survived the treatment, since concentrations we use already induce significantly higher killing than hNGP.

4.1.4 Cost and benefit of hyperinvasion

Hyperinvasion is not a feature of any invasive enteric bacteria. In contrast to *Shigella*, hNGP does not cause hyperinvasion of *Salmonella* (data not shown). This is consistent with the observation that Salmonella modifies its LPS to make it less negatively charged which, in turn, leads to less efficient binding of AMPs (Vaara et al., 1979). In contrast to *Shigella* that resides exclusively in the intestinal epithelium, *Salmonella* disseminates rapidly through the bloodstream soon after oral inoculation (Mastroeni et al., 2009). It is interesting to speculate that *Salmonella* probably evolved to express a LPS that does not allow hyperinvasion, since it can disseminate before neutrophils arrive to the site of infection, but it makes it less sensitive to AMPs. Probably, *Shigella* is under two opposing selective pressures during infection of its host. On one side, binding of antimicrobial proteins and peptides at higher concentrations kills invading bacteria. Although a more negatively charged LPS would increase AMP binding and therefore

invasion, it would also increase *Shigella* susceptibility to these proteins. On the other hand, without binding these proteins, invasion efficiency is much lower. After binding of AMPs from the neutrophil granules, *Shigella* escapes into the safe niche of the epithelial cell cytosol, where it cannot be attacked anymore. Therefore, *Shigella* LPS might have evolved to maintain the balance between binding of AMPs to promote invasion and killing.

4.2 Inhibition of IL-8

Expression and secretion of IL-8 together with other proinflammatory cytokines is crucial for neutrophil recruitment into infected tissue. Since neutrophils are able to kill *Shigella* in contrast to macrophages and epithelial cells, downregulation of IL-8 secretion (Figure 17) in infected epithelial cells provides an excellent evolutionary advantage. However, a limited induction of inflammation may be beneficial for *Shigella*, since this leads to a limited recruitment of neutrophils to the site of infection including hyperinvasion and opening of the epithelial barrier. The observed IL-8 inhibition shuts of further neutrophil recruitment after hyperinvasion occurred and neutrophils are not required anymore for successful infection of the epithelium. Three different mechanisms are possible for IL-8 downregulation.

4.2.1 Inhibition of IL-8 by induction of cell death

First, high numbers of intracellular bacteria caused by hyperinvasion may cause accelerated cell death. This hypothesis is supported by the observed loss of membrane integrity over time by two different assays in an hNGP dependent manner (Figure 19). Sytox uptake, a small DNA dye, can be observed as early as three hours after infection. The higher the number of intracellular *Shigella*, the earlier sytox uptake can be observed. Using another assay, significant release of the intracellular enzyme LDH into the supernatant can be observed about 1 hour after sytox uptake. The LDH

molecule is larger than Sytox (25-40 kDa depending on the isoform). This indicates a progressive loss of membrane integrity leading to larger holes in the plasma membrane allowing release of larger molecules into the extracellular space. These data indicate necrosis as the ongoing cell death mechanism. This is further substantiated by the failure to detect any induction of apoptosis in cells infected with hNGP treated or untreated *Shigella* (Figure 20). We did not detect apoptosis at 1 and three hours after infection with any hNGP concentration. There is no apoptosis induced while necrosis starts to occur. However, we did not check later time points at which necrosis is more pronounced. While it is tempting to assign IL-8 inhibition to the accelerated induction of cell death, several arguments speak against this as the responsible mechanism. We showed that - in contrast to Erk and p38 - Jnk kinase is not inhibited with increasing hNGP concentrations but rather activated stronger (Figure 18). In addition, IL-8 secretion is measured at 3 hours post infection. At this time point we just start to observe cell death in infected cells and inhibition of IL-8 release already took place. However, losing the integrity of the plasma membrane is the last step during cell death and it is difficult to predict how infection influences mRNA transcription and protein synthesis until leakage occurs.

4.2.2 IL-8 inhibition mediated by TTSS effectors

As a second possibility, several TTSS dependent effectors have been reported to inhibit proinflammatory responses of *Shigella* infected cells. These effectors, such as OspF (Arbibe, et al., 2007; Li, et al., 2007) and OspG (Kim, et al., 2005) are able to inhibit activation of NF-kB and MAPK. We could not detect any changes in IL-8 inhibition when cells were infected with hNGP treated *Shigella* lacking either of these effectors. This indicates that IL-8 inhibition was independent of these two effectors. In addition we analyzed IL-8 inhibition by hNGP treated mutants in MxiE, a transcription factor controlling the release of all so called second-effectors

of *Shigella* (Mavris, et al., 2002; Mavris, et al., 2002). This second wave of secreted proteins includes OspF and OspG, but also many more TTSS effector proteins with yet unassigned functions. The hNGP treated *mxiE* mutant readily inhibited IL-8 secretion after infection. This excludes second wave effectors to be involved in hNGP mediated IL-8 inhibition. At this point, there are no more candidate effectors to be tested. An alternative to this candidate approach could be a transposon mutagenesis of *Shigella* screening for mutants impaired in IL-8 inhibition. This could be done in 96-well-format, since the invasion assay can be downscaled and the ELISA for IL-8 is very sensitive. However, since there are other possible mechanisms not involving *Shigella* effectors, these should be ruled out before starting this screen.

4.2.3 Masking or degradation of Nod ligands

Third, detection of peptidoglycan fragments usually shed off by *Shigella* during infection (Nigro, et al., 2008) might not be detected by the intracellular sensors Nod1 and Nod2. Granular proteins on the surface of *Shigella* could mask PGN molecules or degrade them, rendering them inactive. Mammals produce four known PGN recognition proteins (PGRP) (McDonald, et al., 2005). These proteins bind to PGN and degrade them but lack signaling capabilities. One of them, PGRP-S, is present in the granules of neutrophils (Liu, et al., 2000) and binds to gram-negative as well as gram-positive PGN. It has antimicrobial activity and colocalizes with NETs (Cho, et al., 2005). If PGRP-S has PGN degrading activity is unclear. The fact that it binds PGN makes it an interesting candidate for IL-8 inhibition. PGRPs might reduce the proinflammatory potency of PGN in the host either by binding and therefore masking PGN fragment or by degradation into non-activating fragments. Taken together, Nod1 and Nod2 ligands might not be present in sufficient amounts to activate their targets. Finally, we observed that IL-8 secretion was inhibited in cells in which IL-8

expression was induced by incubation with TNF-α before infection. Using poly-lysine, complete abrogation of IL-8 secretion could be observed. This could be due to either IL-8 re-uptake into the epithelial cell is induced by an unknown mechanism or inhibition of expression and secretion is achieved very early after infection.

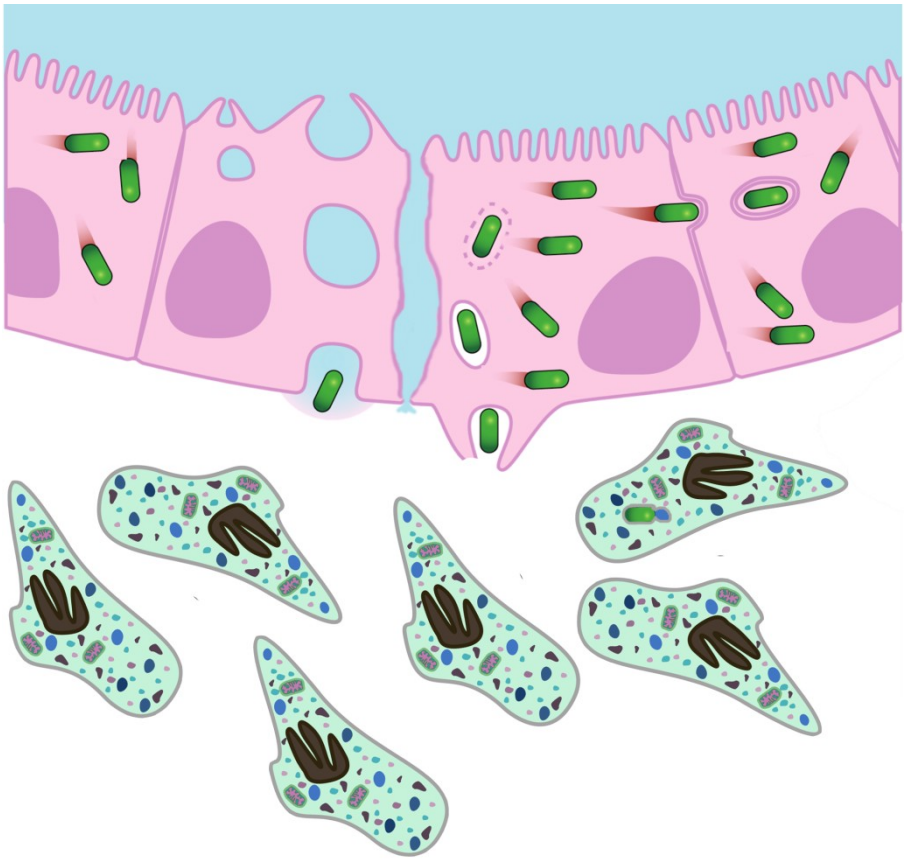

Figure 24: Current model of hyperinvasion

First, few *Shigellae* infect epithelial cells from the basolateral side. After their detection by Nod1 and Nod2, IL-8 expression and synthesis is turned on. After initial neutrophil recruitment by IL-8, *Shigella* becomes hyperinvasive leading to massive infection of the epithelium. Then, IL-8 production is shut down preventing further infiltration of neutrophils. Together with hyperinvasion, this inhibition maximizes survival and multiplication of *Shigella* in the host.

4.3 Conclusions and future directions

After initial neutrophil recruitment by IL-8, *Shigella* becomes hyperinvasive leading to massive infection of the epithelium. Then, IL-8 production is shut down. Taken together, hyperinvasion and downregulation of IL-8 allow *Shigella* to efficiently escape the threat of neutrophils and to inhibit recruitment of more neutrophils to the site of infection (Figure 24). *Shigella* makes use of antimicrobial proteins designated to kill it, thereby subverting their function. Since *Shigella* infections stay locally confined, this strategy enables *Shigella* to withstand attacks from the innate immune system until it reached its goal of multiplication inside the host.

Further questions in this project include the complete elucidation of the mechanism of hyperinvasion by measuring the surface charge of hNGP treated wild-type and ΔwaaL *Shigella*. In addition, binding of cationic neutrophil granular proteins to the ΔwaaL mutant should be enhanced, mediating the increased hyperinvasion observed. Finally, a detailed analysis of the Nod1 and NF-kB pathway will provide clues at which level it is inhibited by infection with hNGP treated *Shigella*. This will enable us to determine the exact mechanism of IL-8 inhibition.

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Appendix

Abbrevations

A	adenosine
Å	Angstrom
α	anti
AP-1	activator protein-1
BPI	Bactericidal / Permeability Increasing Protein
BSA	bovine serum albumin
C	cytosine
CARD	Caspase Activation and Recruitment Domain
DMEM	Dulbecco's modified Eagle medium
DNA	desoxyribonucleic acid
ECM	Extracellular Matrix
EDTA	ethylene diamine tetraacetate
ERK	extracellular signal regulated kinase
FCS	fetal calf serum
G	guanosine
HEPES	2-[4-(2-hydroxyethyl)-1-piperazino]-ethansulfonic acid
HSA	human serum albumin
IκB	Inhibitor of kB
IkK	IκB kinase
IL-1β	Interleukin 1 beta
IL-8	Interleukin 8
IL-18	Interleukin 18
Ipa	Invasion plasmid antigen
Jnk	jun N-terminal kinase
kb	kilobase

kDa	kilo Dalton
LB	Luria-Bertani medium
LBP	LPS binding protein
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MAPKK	mitogen activated protein kinase kinase
MAPK	mitogen activated protein kinase
M-cell	microfold cell
MOI	Multiplicity of infection
MPO	Myeloperoxidase
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NET	Neutrophil Extracellular Trap
NF-κB	nuclear factor κB
NLR	Nod-like receptor
nm	nanometer
Nod	nucleotide oligomerization domain
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PFA	Paraformaldehyde
PGN	peptidoglycan
P.I.	Point Isoeelectrique (isolectric point)
PMA	Phorbol Myristate Acetate
PMN	Polymorphonuclear cells
RNA	ribonucleic acid
ROS	Reactive Oxygen Species
RPMI	medium developed at “Roswell Park Memorial Institute”
SDS	sodium dodecylsulfate

SNARE	Soluble N-ethylmaleimide-sensitive fusion (NSF) factor attachement protein receptor
Spp.	*species pluralis*
T	thymidine
TAB	TaK1 binding proteins
TNF-α	Tumor Necrosis Factor α
TIR	TLR-interleukin-1 receptor
TMB	tetramethyleneebenzifine
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRIF	TIR-containing adaptor inducing interferon β
Tris	Tris(hydroxymethyl)-aminomethan
TTSS	Type Three Secretion System
Ub	ubiquitin

Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde. Der Inhalt der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 19.06.2002 ist mir bekannt.

Björn Eilers Berlin, den